Scanning Electron Microscopy of the Early Life Stages
of the New Zealand Yellowfoot Paua, *Haliotis australis* and Factors Affecting Settlement.

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Abstract

The purpose of this research was to spawn and settle larvae of the Yellowfoot Paua (Yellowfoot abalone) *Haliotis australis* (Gmelin, 1791) to investigate the early life stages of *H. australis* and the factors influencing settlement of the larvae of this abalone species in a commercial aquaculture context.

Scanning electron microscopy (SEM) was used to investigate the morphological aspects of the development of the life stages of *Haliotis australis* from gametes, veliger larvae, post larvae and juveniles to 60 days post settlement (70 days post fertilisation). The photo micrograph results presented in this thesis represent the first comprehensive SEM micrograph record of the early life stages of *H. australis*.

Settlement experiments tested success of larval settlement on four different diatom biofilm settlement substrates; 8-Day [old] Ungrazed Biofilm, 8-Day Grazed Biofilm (pre-grazed by conspecific adults to produce mucus trails), 1 Day Biofilm and No Biofilm. Half of the experimental replicates were settled with *H. australis* larvae treated with gamma(γ)-aminobutyric acid (GABA) to test the effectiveness of GABA as a chemical treatment to enhance settlement of *H. australis* larvae.

Post larval survival at 33 days post settlement was used to infer the settlement success occurring at the time of settlement (between Day-0 and Day-4 post settlement). Analysis of mean survival data at 33-days post settlement identified that established diatom biofilms pre-grazed with conspecific adults (8 Day Grazed Biofilms) produced higher settlement than one day old diatom biofilm (1 Day Biofilm) and No Biofilm (control) treatments. Established diatom biofilms (‘8 Day Ungrazed Biofilm’) produced higher settlement than ‘No Biofilm’ treatments. The differences observed were statistically significant.

The analysis of mean survival at 33-days post settlement identified that there was no significant difference detected between GABA and no-GABA treatments. However the observed results indicated that 8-Day Ungrazed Biofilms and 8-Day Grazed Biofilms treated with GABA, and untreated (no-GABA) 8 Day Grazed Biofilm settlement surfaces produced the best inferred settlement of *H. australis* larvae when compared to untreated 8 Day Ungrazed Biofilm and both GABA and no-GABA 1 Day Biofilm and No Biofilm treatments.

These results suggest that pre-grazing of prepared diatom film settlement substrates with conspecific adult or juvenile abalone may be employed by hatcheries to ensure the highest rates of settlement in *H. australis* larvae. When the pre-grazing of established diatom biofilms is not a practical option in a larger scale aquaculture context, then the treatment of competent *H. australis* larvae with a GABA solution prior to settlement may be used to enhance the success of settlements onto established diatom biofilms.
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Chapter 1 - General Introduction.

1.1 Thesis Overview and Structure.

The following thesis documents the research that was undertaken to examine techniques and processes used to spawn, settle and raise larvae and post larvae of the New Zealand Yellowfoot Paua *Haliotis australis* for a period of 60 days post settlement. The research examines factors that affect the settlement and early development of *Haliotis australis* and examines the physiology of larval and post larval development using scanning electron microscopy (SEM).

The research was industry based and was conducted as part of a Foundation of Research Science and Technology (FoRST) funded, Graduate Research in Industry Fellowship (GRIF) project. The spawning and experimental work was undertaken at the Rainbow Abalone Limited hatchery and abalone production facility located at Port Taranaki, New Plymouth during the period November 1995 to February 1996. SEM analysis of samples taken during the experimental hatchery work was undertaken at the University of Otago between March and July 1996.

Chapter One of the thesis introduces the research, and reviews relevant scientific literature in regard to the taxonomy, ecology, distribution lifecycle growth and development of abalone, including the aquaculture of abalone with a particular reference to *Haliotis australis*.

Chapter Two documents the materials and methods used during this research to spawn, raise and monitor the growth and development of larval and post larval abalone; describes the experimental system in which settlement experiments were undertaken; and describes methods used to analyse the data.

Chapter Three presents the results of the observations of the growth and development of the *H. australis* larvae and post larvae and includes the results of the SEM investigation into development and the results of settlement experiments are presented and analysed.

Chapter Four includes a discussion of the results and the findings of settlement experiments and recommendations for culture are made with reference to results of relevant experimental investigation and scientific literature related to *H. australis* published since 1995.

Chapter 5 presents a final overview of the potential for the culture of *H. australis* in New Zealand, and highlights some areas of interest for future research and closing comments.
1.2 Introduction

The New Zealand Yellowfoot Paua *Haliotis australis* (Gmelin, 1791) has been identified as having a high commercial value (McShane, Mercer & Naylor, 1994a) and has been highlighted as a species with potential for commercial aquaculture (McShane, Mercer, Naylor & Moss, 1994b). This species can grow quickly under the right conditions and has pale coloured flesh that has higher export market acceptance (Jeffs, 2003). Interest in the development of *H. australis* as a species for commercial production in the early 1990’s led to a systematic focus of abalone research at the NIWA Mahanga Bay aquaculture research facility investigating the controlled breeding of Yellowfoot Paua (Hickman, 2009). *H. australis* was considered by researchers as one of the two ‘superior’ abalone species that can be produced in New Zealand. Research at the NIWA Mahanga Bay facility concentrated the four main problem areas encountered by abalone farmers, being brood stock conditioning, settlement, early growth, and on-growing (Tong & Moss, 1992; Moss, 1997, 1998a, 1998b, 1998c, 1999).

Development of New Zealand abalone species for aquaculture has been systematically researched since the late 1980’s with commercial production of abalone in land based aquaculture facilities largely focused on the New Zealand Blackfootabalone *Haliotis iris* (Gmelin, 1791) (Paua). The Blackfootabalone has been successfully cultivated in New Zealand since 1986 in a number of small scale land based aquaculture facilities (Illingworth, 1986; Henriques, 1989; Tong & Moss, 1992; Hickman, 2009; Wright 2011). OceaNZ Blue Limited (established in 2003) is New Zealand’s largest abalone hatchery and grow out facility and is located on the NIWA Bream Bay Aquaculture Park site at Ruakaka, Northland. OceaNZ Blue Ltd. has achieved an annual production of approximately 80 Tons *H. iris* (Blackfoot Paua) in a size range of 87 mm to 102 mm for local and export markets (R. Roberts pers. comm. in Wright, 2011). While there has been successful development and commercialisation of *H. iris* there has also been a long standing interest in the development of the Yellowfoot Paua for commercial aquaculture by many New Zealand abalone culturists.

The early work developmental work on *H. australis* undertaken by Moss and others in the early 1990’s (Tong & Moss, 1992; Moss, 1999) had identified that the larvae stage of Yellowfoot Paua was difficult to settle and suffered high (88-100%) mortality of larvae around the time of settlement (Naylor & McShane, 1997; Roberts & Watts, 2010). Comparisons of settlement and post settlement survival in *H. australis* by Moss (1999) had identified that conspecific mucus trails, diatom biofilms and GABA had all induced larvae to settle and initiate post larval shell growth, while no-biofilm controls had not. It was against the background of interest in the early research on *H. australis* that the 1995 experimental work detailed in this thesis was undertaken. The results of early experimental work undertaken by Moss at the NIWA Mahanga Bay facility in the early 1990’s was published (Moss, 1997, 1998a, 1998b, 1998c, 1999) and it was not until 2003 that further comprehensive experiments examining the
settlement of *Haliotis australis* larvae on a range of settlement cues were undertaken by Roberts and Watts (2010) at the Cawthron Institute aquaculture facility in Nelson.

In 1995, as a result of the interest in development of the commercial culture of *Haliotis australis*, a Graduate Research in Industry Fellowship (GRIF) project was established at the Rainbow Abalone Limited hatchery and grow-out facility located at Port Taranaki in New Plymouth. The purpose of the industry research was to undertake experiments to examine factors affecting the settlement and survival of *H. australis* to establish if existing techniques for abalone culture used in commercial hatcheries in New Zealand and internationally could be adapted for successful commercial culture of *H. australis* at Rainbow Abalone Ltd. The GRIF project examined; a) the inferred settlement of post larval survival *H. australis* at 33-days post settlement against differences in the age of the diatom biofilm used as an initial settlement substrate; and (b) the effects of two methods of settlement induction (namely pre-grazing with conspecific adults, and treatment of competent larvae with a ‘dip’ of \(\gamma\)-aminobutyric acid (GABA)). An important product of the research was the use of SEM to examine larval and post larval stages of *H. australis* including features of the growth and development of juvenile *H. australis* for up to sixty days post settlement. This masters thesis reports the results of that research.
Abalone are generally described as herbivorous archaeagastropod molluscs. All abalone are currently treated as species of the genus *Haliotis* (Linnaeus, 1758) in the monogeneric family Haliotidae (Rafinesque, 1815; Brown & Murray, 1992). Organisms that belong to the Phylum Mollusca are characterised as non-segmented invertebrates with a mantle cavity that typically contains the gills, usually a definite head with a radula, and a muscular foot. The features which make abalone members of Class Gastropoda (Cuvier, 1795) are that they have a single one-piece shell, move by means of a broad muscular foot and show some degree of torsion or asymmetry. Gastropods of Subclass Orthogastropoda (Ponder & Lindberg, 1996) undergo torsion during the veliger larval stage of the life cycle so that the mantle cavity comes to lie at the front of the body, and the nervous system is twisted in a figure eight. Abalone are members of the Order Vetigastropoda (Salvini-Plawen, 1980) which are primitive Prosobranch snails that have no siphon or proboscis and have bipectinate gills (with filaments on both sides of the gill axis). Turban shells and limpets are also members of this order. The characteristics that place all abalone in the Family Haliotidae (Rafinesque, 1815) are the markedly flattened visceral mass and shell in which the shell spire is greatly reduced. A feature of the Family
Haliotidae is the flattened ovoid shell which has a row of holes called respiratory pores. The respiratory current is drawn under the shell and exits through the respiratory pores, carrying faeces, urine and gametes (during reproduction). The scientific literature cites between 30 (Dauphin et al. 1989) and 130 (Cox, 1962) species of abalone worldwide, with some authors citing 210 taxa as being described (Wright, 2011). A recent comprehensive treatment of the Family Haliotidae considers that there are 56 species and 18 distinct subspecies of *Haliotis* worldwide (Geiger & Owen, 2012).

### 1.4 Distribution and Habitat of Abalone

Abalone are found in both hemispheres from arctic to tropic regions, with larger species inhabiting more temperate waters, and the smaller species being found in the tropics and in polar regions. (Hahn, 1989)

Abalone are usually found in the sub-tidal and sub-littoral fringe of exposed rocky coasts, but have been noted to occur in depths up to 400 meters (Lindberg, 1991). Red and brown macroalgae are important food sources for adult abalone (Shepherd & Steinberg, 1991), microscopic algae such as diatom biofilms and crustose coralline algae (*Lithothamnion* spp.) are important recruitment substrates and food sources for juveniles (Akashige et al., 1981; Morse et al., 1984; Moss & Tong, 1992b; Roberts, 2000).

The New Zealand mainland and its outlying islands have three endemic species of abalone (Paua); BlackfootPaua (*Haliotis iris*), Yellowfoot Paua (*Haliotis australis*) (Figure 1) and white foot Paua (*Haliotis virginea*). *Haliotis virginea* is also known as the ‘virgin Paua’ and has four sub-species. All of the New Zealand species of abalone inhabit rocky reef habitat that is close to the shore, where water motion is high and macroalgae is available as a food source (Poore, 1972a; Wright, 2011). The following information presented in this thesis has a particular focus on the New Zealand Yellowfoot Paua species *Haliotis australis*.

### 1.5 Biology and Ecology of *Haliotis australis*.

*Haliotis australis* grows to a maximum length of about 110 mm, inhabits sub tidal crevices and cavities under stones. *H. australis* actively and selectively grazes both drift algae and growing red and brown sea weeds (Poore, 1972a). *H. australis* is a very mobile species and feeds at night (Morton and Miller 1968), which is in sharp contrast to the generally sedentary *Haliotis iris*, which is rarely seen moving in the field. *H. australis* are most abundant from below the low tide mark to about four meters (Poore, 1972a).
1.5.1 Feeding and Growth in *Haliotis australis*.

Laboratory experiments undertaken by Poore (1972c) showed that *Haliotis australis* was a very active feeder preferring red seaweeds (*Hymenocladiaceae lanceolata* and *Pterocladia lucida*) over the brown algae (*Lessonia variegata* or *Macrocystis pyrifera*). Those *H. australis* that were grown on red seaweed grew faster than on either of the brown seaweeds trialed (Poore, 1972c). Wilson (1987) found that the red seaweeds, or rhodophytes, were the most preferred group of algae consumed by adult *H. australis* and that green and brown algae, chlorophytes and phaeophytes respectively, were the least preferred species. Wilson (1987) also found that a mixed diet of these algae produced the fastest shell growth and largest increases in wet weight in *H. australis*.

Naysmith (2000) found that the duration of photoperiod affects the feeding rate in *H. australis* with feeding rates at their greatest under conditions of constant darkness. The feeding rates of both *H. australis* and *H. iris* were both identified as being inversely correlated with day length. Day length may play an indirect role in conditioning abalone by making available more dark hours for feeding (Naysmith, 2000).
Small Yellowfoot Paua are rarely found in the wild, so little is known about their natural growth rates. In culture the growth of post larvae and early juveniles is slow, and in experiments at the NIWA Mahanga Bay hatchery in Wellington juvenile *H. australis* were recorded taking about seven months to reach a mean size of 7 mm in length. When juveniles reached 7 mm in length the growth rate was observed to increase, only taking another four months for them to double their length (Moss, 1998c). When juveniles reach 7 mm they are also able to start feeding on seaweeds or artificial diets without suffering reduced growth rates. The same *H. australis* juveniles were measured to reach a mean size of 15 mm after one year and 35-40 mm after two years when fed on seaweeds at Mahanga Bay in Wellington at ambient seawater temperatures (Moss, 1998c).

### 1.5.2 Shell Colour and Diet

Diet may have an effect on the shell colour of some *Haliotis* species (Leighton, 1961; Olsen, 1968). The internal shells of *H. australis* are consistently white except for some pink tinges in small specimens and diet seems to have little effect on the shell colour of this species (Poore, 1972a). Colour bands appear intermittently in the same position of the growth rings. The external colour of the shell is dependent on the type of food consumed (Leighton & Boolootian, 1963) with pigments thought to result from the metabolism of waste products (Comfort, 1949, 1951). Variation in the diet of *H. australis* can cause marked differences to the external shell colour as seen in Figure 1 where visible colour band have been caused by changes from natural seaweed diets (brown) to artificial diets (blue green). The brood stock abalone supplied by NIWA Mahanga Bay for this research had this characteristic colour banding.

### 1.5.3 Movement of *Haliotis australis*.

Little information is available on the movement of juvenile *H. australis*, since they are rarely found inter-tidally (as are small *H. iris*) and the few that are seen sub-tidally are generally located in crevices or under stones (Poore, 1972b).

*Haliotis australis* is an active species, often seen moving over plants and substrate during daylight and moving about at night. Since little food reaches this species in its crevices, a foraging and homing behavior seems likely. There is negligible long-term movement of *H. australis* adults and there are definite home sites (Poore, 1972b).

Larger older adult *H. australis* have been observed to inhabit deep worn depressions in the rock surface and often when removed cannot clamp shell down on to a flat surface. Others have a characteristically worn shell edge, which fits the surrounding rock closely, which suggests a lack of natural long-term movement (Poore, 1972b). Both juveniles and adults of *H. australis* use the same habitats, narrow sub-tidal crevices or under large flat boulders (Wilson, 1987), so little migration of juveniles is likely (Poore, 1972b).
1.5.4 Reproduction in *Haliotis australis*

*Haliotis australis* is dioecious with no discernable difference between the sexes except for the colour of the gonad. The gonad is a brown-purple colour in females and a creamy white colour in males. A study of the gonads of *H. australis* populations at two sites in southern New Zealand found that female *H. australis* have primary and mature oocytes at 61 mm body length and the smallest male to have sperm present was at 65 mm in length (Wilson & Scheil, 1995). An examination of the fecundity of *H. australis* in the same study found that adult females between 80-90 mm in length produced approximately 2.7 million eggs during a spawning event.

The Yellowfoot Paua has a seasonal reproductive cycle which may have two spawning periods each year (Poore, 1973; Wilson, 1987). The first potential spawning period is in the autumn months of March / April and a second spawning period is in the spring around September (Wilson, 1987). However in some years *Haliotis australis* may not spawn at all (Poore, 1973). Kabir (2001) observed that the reproductive cycle of an *H. australis* population at Warrington in Otago was annual and synchronous among sexes. Spawning in that *H. australis* population occurred in summer and autumn in both 1997 and 1998. Another major spawning was recorded in the spring of 1996, but this was not repeated in 1997 (Kabir, 2001). It is likely that there is some geographic variability in the timing and or frequency of spawning due to factors such as water temperature, food availability and environmental stimulus (Rogers-Bennett et al., 2010).

Adult abalone are likely to be triggered to initiate spawning by environmental factors which may include rapidly changing water or air temperatures, changes in water chemistry, storms, or the release of gametes by other individuals (Campbell, 2000). A study of reproductive periodicity and spawning in two New Zealand abalone (*H. iris* and *H. australis*) found no significant correlation between gonad maturation and day length, or sea temperature. However drops in monthly gonad index (indicating spawning events) in both species was correlated with sustained large (3m) swell events indicating that wave events may trigger spawning in these species (Naysmith, 2000).
1.5.5 Lifecycle of *Haliotis australis*

The stages of the lifecycle of *H. australis* is similar to all other abalone species and is summarized in the lifecycle diagram shown in Figure 2. The stages of the lifecycle of abalone can be isolated into four main periods: (1) Spawning and fertilisation; (2) larval development; (3) settlement and metamorphosis; and (4) post settlement growth and development.

![Lifecycle of abalone H. australis](image)

Figure 2: Life cycle of the abalone *H. australis* (not to scale).

1.5.5.1 Spawning and fertilisation

All abalone spawn by ejecting gametes into the surrounding water column. During spawning, adult *H. australis* abalone rise up on their foot, raise their shell and extend their epipodial tentacles prior to suddenly contracting in a smooth powerful motion. The force of the contraction of the foot and shell causes the gametes to be expelled from the gonad. The proximity of the gonoduct near the gills helps ensure that gametes are flushed out through the respiratory pore by respiratory currents to maximize dispersal into the surrounding water column. The presence of spermatozoa in the water column stimulates female *H. australis* to initiate and begin spawning within a few hours. *H. australis* produce large spherical yolk filled eggs approximately 180 microns in diameter which have a distinctive purple colour. The eggs are slightly negatively buoyant and settle in the vicinity of the adult abalone, with the dispersal of eggs largely dependent on water movement. The fertilisation of the egg and sperm occurs externally in the water column.
The large store of yolk within the egg is characteristic of species that have a lecithotrophic larval stage with the yolk store providing the primary source of nutrients for the developing embryo and for the larvae during a non-feeding, free-swimming, development period in the water column.

1.5.5.2 Larval Development

The rate of embryonic and larval development is primarily regulated by water temperature and the fertilised eggs undergo rapid cell division typical of abalone. Development proceeds through the initial embryonic stages of cell division (Figure 3) through morula and gastrula stages to reach the first stage of larval development (trochophore stage) usually within 24 hours.

The trochophore larvae are diamond shaped and have a girdle of cilia called a ‘prototroch’ and a cluster of cilia located on the top of the trochophore called the apical tuft. The action of the cilia of the prototroch beating causes the trochophore to spin within the egg casing, eventually causing the eggs outer membrane of the egg to rupture and allowing the trochophore to hatch out into the water column.

The trochophore are positively phototactic (Huner & Brown, 1985) and the prototroch provides the initial means for trochophore larvae to ‘swim’ and maintain its position in the water column.

As abalone larvae are lecithotrophic and do not actively feed during their pelagic larval development phase, it is likely that the larvae absorb dissolved organic matter (DOM) from the water column during their development (Manan & Jaeckle, 1992). The trochophore, pre-torsion larvae and post-torsion veliger larval stages exhibit negative geotrophic behaviour (Wright, 2011). This behaviour ensures that larvae remain in the relative safety of the water column to ensure dispersal and avoid being predated by benthic filter feeders (Crisp, 1974).

Over approximately 24 hours following hatching, the trochophore continues cell division and develops into a pre-torsion veliger larvae which is indicated by the formation of the larval shell (protoconch) and the development of the velum (Crofts, 1937). The morphogenic event of torsion follows soon after the formation of the larval shell which results in the 180° rotation of the shell and viscera relative to the head and foot of the veliger larvae (Pennington & Fu-Shiang, 1985). Over the next six to twelve days the abalone larvae continue to develop in the water column. Following torsion the post torsion veliger larvae develop an operculum, eye spot, foot and a snout, and the beginnings of the cephalic tentacles (Tong, 1983).
Figure 3: Developmental stages of an abalone:

1 - polar bodies appear on the fertilised egg –15–30 min.; 2 - 2-cell stage; 3 - 4-cell stage - 80 min.; 4 - 8-cell stage - 120 min.; 5 - 16-cell stage - 160 min.; 6 - morula stage - 195 min.; 7 - gastrula stage - 6 hrs.; 8 - trochophore in the egg membrane - 7–8 hrs.; 9 - newly hatched trochophore - 10–12 hrs.; 10 - early veliger - 15 hrs.; 11 - late veliger - 48 hrs.). Adapted from “Training Manual on Artificial Breeding of Abalone (Haliotis discus hannai) in Korea DPR”, FAO 1990. Adapted with permission
Figure 4: Developmental stages of an abalone - continued:
(12 - peristomal larval stage - 6–8 days; 13 - early post metamorphosis post-larva with epipodes; 14 - Post larva at 19 days; 15 - juvenile abalone – 30 day). Adapted from "Training Manual on Artificial Breeding of Abalone (Haliotis discus hannai) in Korea DPR", FAO, 1990. Adapted with permission.

The formation of the operculum enable the larvae to retract their bodies and velum into the relative safety of the larval shell. The post torsion veliger is also referred to as peristomal larval stage (Figure 4). Larvae develop for 10 to 15 days within the water column after hatching until the larvae are competent (ready) to settle on a suitable substrate and begin metamorphosis into post-larval abalone (Moss & Tong, 1992b).

1.5.5.3 Larval Settlement and metamorphosis

‘Larval settlement’ has been defined as the transition from a swimming larva to a crawling, feeding post larva, and is divided into three stages: pedal attachment; initiation of metamorphosis; and development of post-larval shell growth (Roberts, 2001).
When larvae are competent to settle, they begin a ‘crawling larvae’ phase (as identified in the lifecycle diagram in Figure 2) where larvae cease swimming and begin to test the benthic substrate, first attaching to the substrate, crawling along it, and sweeping their snout from side to side.

If the substrate is not suitable, the larvae have the ability to swim away from the surface and drift in the current and attempt to settle again at a different location. If the surface is suitable the larvae will remain settled on the substrate and undergo metamorphosis. The presence of crustose coralline algae (*Lithothamnion* sp.) is known to trigger the settlement and metamorphosis of larval abalone in the wild (Tong, 1982; Roberts, 2000). Metamorphosis involves the loss of the velum and the beginning of the growth of the post larval shell known as the peristomal shell. The settled (metamorphosed) larvae is committed to the benthos and begins feeding soon after. The peristomal shell growth of the settled post larva occurs rapidly over a period of two to three weeks and is a period when significant mortality occurs. The formation of the first respiratory pore occurs at around 30 days post settlement depending on temperature. Following the formation of the respiratory pore survivorship of *H. australis* improves and the post larvae are considered to be a juveniles.

### 1.6 Aquaculture of Abalone

Abalones are cultured in a variety of land and sea based aquaculture systems, to produce abalone juvenile ‘seed’ for reseeding wild stocks (Hickman, 2009) or for the production of small ‘cocktail abalone’, which can be harvested at a size smaller than those abalone that can be legally obtained from wild fisheries. Cultured abalone may also be on-grown and implanted with a nucleus to produce a nacreous pearl (Moss, 2000) which yields not only abalone meat but potentially several high value half pearls at harvest. Abalone is traditionally a highly sought after fisheries product in most cultures and is particularly valued in Asian cultures particularly in China, Japan and Korea. Land based culture of abalone in ‘flow through’ or recirculating systems is very capital intensive, with major costs being the pumping of seawater, the culture facilities and associated equipment and maintenance, abalone feed and labour. The potential for high value yields of abalone meat and other by-products to outweigh the significant costs that are involved in production, continues to sustain interest in the potential development of new abalone species for commercial culture.

Development of abalone as potential species for aquaculture and enhancement of wild populations in New Zealand had been the focus of research since the early 1980’s (Dutton & Tong, 1981; Tong, 1982, Tong et al., 1987). A fledgling New Zealand abalone aquaculture industry had developed in the late 1980’s and early 1990’s and was focused largely on the development of the New Zealand Blackfootabalone *Haliotis iris* (*Paua*) for commercial production. Reliable methods for the production of commercial numbers of the *Haliotis iris*, had already been established by Tong *et al.*, (1987; 1992) and the industry was looking to develop other New Zealand abalone species for commercial culture.
Early success with *H. iris* led to a period of research and development in which *Haliotis australis*, an endemic New Zealand species of abalone possessing a yellow foot (also known as Yellowfoot Paua, ‘Queen’ or ‘Silver’ Paua), was identified as a native abalone species that was worthy of commercial aquaculture. An evaluation programme involving broodstock conditioning, spawning and on growing of *H. australis* was undertaken by New Zealand fisheries and aquaculture scientists from NIWA (formerly Ministry of Agriculture and Fisheries - Fisheries Division) (Maxwell - unpublished GRIF report, 1995; Moss, 1998b, 1998c). Offshore markets for abalone appeared to have a preference for pale coloured abalone flesh. Consequently the Yellowfoot Paua, with its pale coloured foot was considered likely to fetch higher market prices, and it was found that the Yellowfoot Paua had a higher meat yield, size for size, than the Blackfoot Paua, *H. iris* (Moss, 1998a). The Yellowfoot Paua *Haliotis australis* was therefore considered an excellent candidate for further development for aquaculture in New Zealand. However significant challenges had been encountered during early trials with *H. australis* that required further investigation.

Ralph Brown of Crystal Park Marine Farm, (a small scale abalone hatchery and grow out facility located on the coast at Riversdale Beach on the Wairarapa East coast) carried out some early research and development investigating the spawning and culture of *Haliotis australis* with some success (R Brown. personal comm. 1995). Graeme Moss of NIWA, located in Mahanga Bay Hatchery, Wellington, undertook a systematic program of research into *H. australis* in the early 1990’s that included feeding and growth trials and development of brood-stock conditioning methods (Moss, 1998b). Successful culture of hatchery reared Yellowfoot Paua was achieved by Moss (1999) in the early 1990’s and resulted in the production of the first generation of hatchery reared adult *H. australis* that were used as the broodstock for spawning undertaken for this research in 1995.

One of the aims of the fledgling New Zealand abalone industry in the early 1990’s was to develop a reliable method of producing *Haliotis australis* on a commercial scale (Ewing, Pers. comm. 1995). It is against this background of pioneering development of new species that the research at Rainbow Abalone Limited was undertaken.

### 1.6.1 Origins of Abalone Culture

The popularity of abalone or ‘awabi’ in Japan as a revered traditional food source has meant that Japanese scientists, since as early as 1893, have led the world in the research into edible marine invertebrates including the culture of abalone (Ino, 1980).

The successful culture of abalone relies on the control of each step of the culture process from broodstock conditioning, spawning, larval development and grow out. Successful culture requires a good understanding of the life history and habits of the abalone and the processes that control them.
The microscopic size of the early life stages in abalone means that observation of development is difficult for many culturists even with specialised equipment.

1.6.2 Broodstock for Abalone Culture

Unpredictability of spawning and availability of brood stock that are well conditioned (ready to spawn) causes fluctuations in availability of seed available for grow out (Hahn, 1989a). Conditioned brood stock can lead to improvement in the planning of the production cycle in the culture of abalone and allow out of season production to take advantage of seasonality of peak growing conditions and ensure predictable spawning in genetic improvement programmes (Ritar, 2000). Water temperature (Uki & Kikuchi, 1984) and diet (Buchal et al., 1998) are the most important limiting factors in the conditioning of Haliotis spp.

The development of genetic improvement of brood stock is a key area of importance for any developing abalone industry and a focus on producing families of brood stock and selecting brood stock based on growth rates, meat-shell ratios and survival at different developmental stages is recommended (Li, 2000a). Advances in the cryopreservation of sperm offers potential opportunities to allow the fertilisation of abalone species that may spawn asynchronously, and ease the shipment and dissemination of gametes that possess the characteristics of the latest advances in abalone genetics. (Li, 2000b).

Broodstock conditioning is achieved by rearing abalone under constant temperature conditions to induce gonad maturation (conditioning). The time required to bring adult abalone into spawning condition is calculated by measuring effective accumulative temperatures (EAT) (in °C hours or °C days). EAT is the cumulative temperature above a Biological Zero Point (BZP) temperature where the gonad development under conditioning has generally shown a progression of development within the gonad to ripeness. In a broodstock conditioning study of H. discus hannai undertaken by Hahn (1994) abalone gonads were shown to have increasing numbers of gametes, and the gametes developed to a more advanced stages as the EAT increased during the experiment. For H. discus hannai gametogenesis was initiated with onset of EAT and continued until 600 °C-days. The period between 600 °C-days and 900 °C-days was identified as the end of gametogenesis and the start of vitellogenesis in the ovary where the mean oocyte volume rapidly increased after 900°C-days until it reached a maximum at 1500°C-days (Hahn, 1994). When all individuals in the broodstock population are ripe and ready to spawn the EAT has been reached. The ripe individuals are easily able to be induced to spawn, and the spawned gametes are viable and fully mature.

Kabir (2001) found that the natural changes in gonad indices in populations of wild H. australis and H. iris at Warrington in Otago and on Stewart Island showed a strong relationship with the EAT. In a
controlled broodstock conditioning experiment, Kabir (2001) found that most rapid gonad growth was achieved at 15°C water temperature in both *H. australis* and *H. iris*. The BZP temperature was determined to be 5.02°C for *H. australis* and 6.21°C for *H. iris* and the EAT required to produce a fully ripe and spawnable gonad in *H. australis* was calculated to be ≥ 1400°C-days and for *H. iris* ≥ 2700°C-days (Kabir, 2001). The research also found that visual gonad assessment criteria were found to be a good alternate for histological assessment without sacrificing the abalone and established that the BZP for larval development in *H. australis* was 5.02°C, identical to the BZP for gonad development (Kabir, 2001).

Harrington (2000) identified that *H. australis* broodstock that had been conditioned with artificial diets (MAKARA) and a mixture of artificial diet and fresh seaweed (*Macrocystis* sp. and *Gracillaria* sp.) produced faster and more consistent gonad development, and that may contribute to better spawning success and larval survival.

### 1.6.3 Spawning of Abalone

Spawning periodicity and duration varies both within and between abalone species (Shepherd & Laws, 1974; McShane et al., 1988; Tutschulte & Connell, 1981), and variability of the spawning period from year to year makes it impossible to determine the precise spawning period (Hahn, 1989a). In the wild *Haliotis australis* was found to have two main spawning periods, occurring between September and October and with a second spawning period in April (Poore, 1972b).

Natural spawning is not completely understood and may be triggered by a sudden increase or decrease in water temperature, exposure to air during low tide, changes in photoperiod, lunar cycle, and release of gametes from other individuals in the population (Hahn, 1989e). Even complex changes in the water chemistry that may occur during storm events in surf zones and along rocky coasts has been speculated as triggering spawning and natural spawning events may be influenced by a combination of the stimuli mentioned above.

The aquaculture of abalone is dependent on the reliable production of gametes and the controlled fertilisation to ensure the synchronous development of larvae. The means by which abalone culturist achieve this control over this critical step in the life cycle of *Haliotis* sp. is termed ‘spawning induction’.

Research into the artificial culture of abalone began in Japan in 1893 when Kishinoue first attempted to obtain viable gametes from abalone by gamete stripping. The hand stripping of abalone is not usually successful with zygotes developing to the two – four cell stage before dying (Ino, 1980). Even if eggs are mature, they are unable to be fertilised when taken directly from the gonad before undergoing the final maturation step that occurs immediately before spawning (Boolootian et al., 1962). Artificial
induction of abalone was unsuccessful until Murayama (1935) added sperm to a bucket containing mature females and induced them to spawn. However this method did not allow for the controlled and synchronous fertilisation of eggs that is required for the hatchery production of larvae.

Carlise (1945) experimented with the use of desiccation treatments to induce spawning in the Californian abalone *Haliotis rufescens*. Ino (1952) was the first researcher to experiment with thermal shock treatments to induce spawning in the Japanese abalone *Haliotis gigantea* and *H. discus hannai*. Kikuchi & Uki (1974a, 1974b, 1974c) subsequently discovered that the most simple and reliable method for the induction of spawning in *Haliotis* sp. was the irradiation of seawater by ultraviolet (UV) light. Morse et al. (1977) experimented with use of a ‘chemical induction’ treatment of a solution of hydrogen peroxide and sodium hydroxide to induce *Haliotis rufescens* to spawn. The chemical induction technique has been found to be the most successful method for the reliable induction of spawning in the New Zealand species *Haliotis iris* (Tong et al., 1992). The chemical induction technique described by Tong et al. (1992) is also effective for spawning of conditioned *H. australis*.

1.6.4 Larval Rearing of Abalone

Larval production is one of the key elements in the commercial aquaculture of *Haliotis* species. The culture of larvae in specialised hatchery tanks provides controlled, stable conditions during the larval development process. Controlled conditions helps to ensure the survival and successful development of large numbers of larvae needed to seed the prepared diatom biofilms that are routinely used for commercial juvenile production.

The larval development process has been well studied for a number of abalone species (Hahn 1989d). The detailed research on the larval development of *Haliotis discus hannai* by Seki and Kan-no (1977) has been the template from which subsequent researchers have modeled studies of other *Haliotis* species. There are 41 distinct larval stages, with recognisable external features, from fertilisation until initiation of metamorphosis in *Haliotis discus hannai* (Seki and Kan-no, 1977). Key stages of the development of larvae are the formation of the trochophore, pre-torsion veliger, post-torsion veliger and crawling larvae that exhibit advanced radula development and substrate ‘testing’ behaviors prior to settlement and initiation of metamorphosis.

Larval development is a period of high mortality in the culture process, and is prone to both bacterial and ciliate out breaks in rearing tanks (personal observation). Contamination and high levels of bacteria can be lethal to molluscan larvae (Lewis et al., 1988). Larval culture ideally takes place in clean hatchery conditions in larval rearing system that utilises seawater that has been passed through a 1 µm filter and which has been sterilised by exposure to ultra violet (UV) light. Throughout the rearing process, the bottom of rearing tanks must be routinely siphoned to remove unhatched eggs and dead or
undevoloped larvae. Water in the larval rearing tanks is required to be changed several times a day and treated with antibiotics, to limit bacterial out breaks during the larval development process (Tong et al., 1992).

1.6.5 Settlement of Cultured Abalone Larvae

One of the most challenging periods for abalone culturists is that which involves the settlement of larvae and early post-larval survival. It is a critical stage in the microscopic life history of abalone which involves the termination of the dispersive, lecithotrophic (non-feeding), planktonic larval stage of the abalone and its transition to a bottom dwelling post larva that feeds on benthic diatoms and associated biofilms. The transformation from larva to post larva involves two distinct processes: settlement followed by metamorphosis (Crisp, 1974; Chia, 1978; Bickell & Chia, 1979; Hadfield, 1984; Slattery, 1992). It is in this period of the culture process that a high rate of mortality of both pre-settlement and post settlement larvae occurs with mortalities from 90 to 99% (Searcy-Bernal et al. 1992a).

To ensure a successful settlement and survival of larvae most abalone hatcheries use a surface diatom biofilms as the preferred surface on which to settle larvae (Roberts et al., 2000). Some also pre-graze the diatom biofilm with adult abalone of the same species prior to settlement to produce mucus trails, which may induce settlement (Grant, 1981). Roberts et al. (2000) found that fast growing benthic diatoms generally dominate un-grazed biofilms and settlement on these biofilms is variable and often low. Few diatom strains are consistently good for settlement and diatom strains that are excessively mobile, or form three dimensional colonies, can prevent successful settlement (Roberts et al., 1999, 2000). Some abalone culturists use treatments of chemicals as ‘settlement inducers’ to trigger and enhance settlement (Morse et al., 1977). In most abalone hatcheries the successful settlement and early development of larval abalone is a critical factor that requires sufficient numbers juveniles to be produced following each spawning event to stock grow out facilities in order to maintain production and sustain an established or developing market.

Abalone larvae swim for several days before becoming competent to transform into a crawling benthic snail (Roberts, 2000a). Settlement is triggered by external cues and knowledge of these cues is critical in abalone culture where complete, rapid and predictable settlement is desired but seldom achieved (Searcy-Bernal et al., 1992a; Roberts et al., 1998).

Abalone veliger larvae can be induced to settle within culture systems. A successful settlement induction is assured by raising healthy larvae and allowing time for the larvae to reach a proper stage of development (Moss & Tong, 1992a) before they are transferred to settlement tanks and induced to settle on prepared strata (plates or tank walls) (Hahn, 1989c). Larvae that are ready to settle are deemed to be ‘competent larvae’.
1.6.5.1  Competent abalone larvae

Experiments and observations of settlement in *Haliotis discus hannai* made by Seki and Kan-no (1981a) observed that rotating the foot and securely clinging to the substratum indicated that the larvae are ready to settle. Competent larvae were seen to exhibit two settling behaviors: Sometimes larvae swam close to the substratum, with their foot parallel to the substratum, and settled softly when the bottom of the foot touched the surface. At other times the larvae collided with the substratum when the foot was inside the shell, and after the collision with the substratum, velar cilia begin beating, the foot was extended from the shell and it twisted until it attached to the substrate and the larva righted itself. Seki and Kan-no (1981a) identified that it took approximately 30 minutes from the initial ‘testing’ of the substrate to settlement and that the settled larva secreted mucus from its foot to increase the strength of the attachment to the substrate. Seki and Kan-no (1981a) also found that settled larvae were so firmly attached to the substrate that a stream of water from a pipette could not remove them from the surface.

After settling the veliger larvae crawl continuously and beat the velum occasionally. The veliger can move very quickly on its foot, traveling up to 200 microns in three seconds (Hahn, 1989d). Crawling becomes progressively slower and then finally stops. At this point the larvae do not move except to change direction and begin testing the substrate. Larvae show a characteristic behavior of raising the back of the shell, until the front of the shell touches the surface allowing the cephalic tentacles sweep the surface of the substrate (Seki & Kan-no, 1981a).

If the substrate is unsuitable the larvae may return to the water column and resume the search for a suitable settlement site (McShane, 1992), however if the substrate is suitable for settlement the larvae shed their velum and lose their ability to swim off the substrate. Settlement is considered complete when the larvae displays active crawling behavior on the settlement substrate and the loss of the velum (Roberts et al., 1998).

In a hatchery it is important for larvae to settle quickly and synchronously, otherwise unsettled larvae are lost from the settlement tanks when water and airflow is restored. Some species of *Haliotis* settle rapidly, while others, like *H. australis*, retain the ability to return to the water column and test other substrates. The ability of larvae to return the water column results in asynchronous settlement of larvae (Moss, 1998b).

1.6.5.2  Settlement induction in Abalone

Larvae test the substrate with their cephalic tentacles but may not start feeding on diatoms for several days (Moss, 1999). It is likely that the post-larvae, utilises the last of its yolk reserves (stored under the larval shell) and dissolved organic matter in the water column during the early days of post settlement development (Manahan & Jaeckle, 1992). During this period where larvae are described as “creeping
larvae” if metamorphosis has not yet occurred the larvae may re-enter the water column and can potentially be lost from the culture system. To ensure successful settlement of *Haliotis australis* settlement inducers may be used to enhance settlement and to ensure synchronised development of post larvae.

### 1.6.5.3 Natural Settlement Substrates - Crustose Coralline Algae (CCA)

In the wild juvenile abalone are found closely associated with crustose coralline algae (McShane et al, 1988; Roberts, 2001a). Morse et al. (1979) identified that crustose coralline algae (CCA) such as *Lithothamnion* spp. could induce settlement behavior in *Haliotis* spp. The CCA not only have chemicals that induce settlement, but also provide an initial food source for the developing larvae (Garland & Cooke, 1985). The surface of CCA have associated micro-algae and bacterial biofilms that are thought to be the initial food sources for newly settled abalone larvae and the textured contours of the surface of CCA also provide shelter and camouflage for the developing larvae (Roberts, 2001a). CCA have been identified as a preferred natural settlement surface for New Zealand abalone species and provide a suitable habitat and food surface for settling larvae. CCA contain an inductive chemical that trigger the larva to settle, attach to the substrate and lose its velum; beginning metamorphosis (Roberts, 2001a). Morse et al. (1979) isolated a chemical from the CCA that was found to be similar to the human neuro transmitter gamma(γ)-aminobutyric acid (GABA). The inducer purified from the cells of *Lithothamnion* spp. mimics the activity of GABA (Roberts, 2001a). Morse (1979) and his colleagues successfully developed a process to induce larvae of the abalone *H. rufescens* to settle using a chemical treatment containing GABA.

PVC half pipes encrusted with CCA in addition to inoculating settlement tanks with mixed diatoms (preferably of *Navicula* species.) successfully used as a settlement surface by Ralph Brown of Crystal Park Marine Farm to settle *H. australis* larvae (Brown, pers. com., 1995). The encrusted PVC half pipes provide shelter and shade for the developing abalone and allows larvae to be easily transferred to other tanks without stressing the sensitive larvae. However in the commercial aquaculture of abalone the use of coralline algae as a settlement substrate is not widespread and GABA treatments, diatom biofilms or pre-grazed conditioned diatombiofilm plates are most commonly used to induce settlement (Freeman, 2001).

### 1.6.5.4 GABA – A chemical settlement induction cue

The use of GABA as a chemical treatment to induce the settlement of abalone larvae is widely used in hatcheries producing *Haliotis rufescens* in the United States (Morse, 1991) and Mexico (Searcy-Bernal et al., 1992b). In the hatchery, GABA is used to mimic the settlement inducers found in coralline algae.
GABA treatments cause larvae to settle rapidly by causing the arrest of the swimming velum (Akashige et al. 1981, Hahn 1989). Early use of GABA treatments as a chemical cue to settle larvae usually required the use of antibiotics in settlement tanks to aid post settlement survival. However the use of antibiotics may affect bacterial growth in the settlement biofilm that is the food source for the developing larvae (Roberts, 2001a).

1.6.5.5 **Diatom biofilms as settlement substrates**

Many abalone hatcheries experience inconsistencies in seed production (Roberts et al., 1998) which is often due to variations of the settlement substrate that are used as a food source for post larval growth. Hatcheries tend to use a range of methods to enhance the settlement of larvae and the use of prepared diatom biofilms is one of the most widely used methods that hatcheries use to settle larvae. Biofilms used for hatchery production of abalone larvae should contain diatoms of a size range (5-10 μm) suitable for ingestion by larval abalone (Norman-Boudreau et al., 1986).

In addition to diatoms, a biofilm will contain many strains of bacteria, fungi and other microbes, as well as a variable amount of extracellular secretions, and a wide array of organic molecules (Roberts, 2001a). Roberts et al. (1998) found that metamorphosis of *H. iris* was reduced by 60% if the inducing diatom film was grown in the presence of antibiotics.

Benthic diatoms are the primary food source for abalone prior to consuming macroalgae (Tomita and Tazawa, 1971; Kawamura, 1996; Kawamura et al., 1999). Diatom species that attach tightly to the substrate are beneficial for post larvae larger than 1 mm, as it results in the larvae being able to use its radula to rupture the siliceous cell wall of the diatom (Roberts 2001a). The breaking open of the diatoms siliceous frustule (skeleton) allows digestion of the intracellular contents of the microalgae (Kawamura et al., 1995). Daume et al. (2000) found that tightly attached, *Navicula* species of diatom, produced larger post larvae and greater survival in the first 70 days post settlement in the Australian abalone *Haliotis rubra*.

Unattached diatoms ingested by early post larvae may pass through the gut intact. It is presumed that the metamorphosing larvae digest the extra-cellular secretions of the diatoms (and also the film of bacteria, fungi and other microorganisms that live on it) before excreting a pseudo-faeces that contain the intact diatoms. It is important for hatcheries to maintain a hardy base of tightly attached micro-algae as a base for settlement plates on which to settle larvae.

The macroalgae *Ulvella lens* is a species that has proven to be useful in Japanese hatcheries as a food source for post larval and juvenile abalone (Roberts, 2000). *Ulvella lens* is a crustose green algae that grows as prostrate rosettes on hard surfaces (Roberts, 2001a). The use of *Ulvella lens* as a settlement surface has resulted in the stabilization of the hatchery production process, resulting in higher survival
and larger juveniles in Japanese abalone, *Haliotis discus hannai* (Takahashi & Koganezawa, 1998). Australian abalone research using *Haliotis rubra* has also indicated that germings of *Ulvella lens* produced settlements of up to 52% of larvae (Krsinich et al., 2000). Tomita and Tazawa (1971) suggest that juveniles move through a transition from a biofilm dominated diet to a macroalgal-dominated diet when they reach a shell length of ~5mm (Kawamura 1996).

Growth of macroalgae such as *Ulvella lens* on settlement plates helps to alleviate the difficult period of maintaining adequate food to juveniles in nursery tanks which is important during the transition when microalgal biofilms and macroalgae are under grazing pressure from larger juveniles and also from copepods. *Ulvella lens* appears to be resistant to grazing by copepods (Krsinich et al., 2000).

### 1.6.5.6 Pre-grazing diatom biofilms with conspecific adults to induce settlement.

Japanese settlement techniques utilise the preparation of settlement tanks by growing a diatom biofilm in settlement tanks (Grant, 1981). Japanese culturists pre-graze the tanks with conspecific adults for several days before introducing competent larvae to the settlement tanks. Pre-grazing creates space amongst the diatom/bacterial biofilm and leaves trails of mucus from the foot of the adult (Seki & Kan-no, 1981b). The mucus products from the adult conspecifics, or the bacteria that colonise them, was thought to contain biochemical cues that induce settlement and may also be an important early food source for the developing post-larvae. Recent studies by Laimek *et al.* (2008) have confirmed that GABA is the settlement-inducing effector molecule contained within abalone mucus, with GABA identified as being present in the nerves and epithelial cells of the abalone foot. HPLC analysis of dried abalone mucus samples revealed a mean concentration of 0.68 μM GABA was present and bioassays using both dry and fresh mucus strongly promoted induction of larval settlement (Laimek *et al.*, 2008). Pre-grazing with conspecifics may also change the species composition of the biofilm over time, leaving the preferred firmly attached diatoms and consuming 3-dimensional colonies of diatoms that may hinder post larval survival (Roberts *et al.*, 2000).

### 1.6.6 Metamorphosis in Settling Abalone Larvae

In New Zealand abalone species the combined use of settlement inducers may have synergistic effects in inducing a settlement response and triggering metamorphosis (Roberts & Nicholson, 1997). Metamorphosis is a critical stage in the life history of marine invertebrates and occurs during the transition from the dispersive planktonic stage to the benthic juvenile form.

The transformation from larva to juvenile involves two distinct processes: settlement followed by metamorphosis (Crisp, 1974; Slattery, 1992). Settlement has been described as a behavioral change, typically characterised by the active searching for, and orientation to, certain environmental factors such
as conspecifics, prey species, algae, sediments, and inorganic cat ions (Crisp, 1974; Hadfield, 1978; 1984). Metamorphosis is a non-reversible phenomenon that involves dramatic anatomical and physiological changes in larvae, ultimately yielding the juvenile form (Bonar & Hadfield 1974; Slattery, 1992).

Metamorphosis is well documented by abalone researchers, but the time at which metamorphosis is complete is not well defined and the period of time that metamorphosis takes is not well described in the literature. There are two schools of thought with regard to metamorphosis in abalone:

Hahn (1989) provided an alternative view that metamorphosis is not sudden, but is a gradual and progressive development of the foot, mouth, digestive gland, circulatory system and sensory organs until the juvenile form is visible. Abalone are considered juveniles at the ‘notch’ stage, which is signalled by the formation of the first respiratory pore (Leighton, 1972, Hahn, 1989). This interpretation of metamorphosis is defined as the period of development with rapid and varied growth, beginning with loss of the velum and ending with the formation of the first respiratory pore (Hahn, 1989).

Many researchers indicate metamorphosis is initiated with the loss of the velum and complete with the formation and growth of the peristomial shell after settlement (Roberts, 2001b). Seki and Kan-no (1981a) considered metamorphosis to be a very short period (complete in less than 24 hours). This short period of metamorphosis has been adopted by leading abalone researchers, Roberts, Kawamura, Daume and Searcy-Bernal (Roberts, 2001b).

1.6.7 On-Growing Juvenile Abalone in Culture.

It soon becomes difficult or costly to maintain juvenile tanks with a diatom food supply, because of the exponentially increasing food demands of juvenile abalone (Roberts et al., 1999). Fine grained powdered diets may be used, first as a supplementary diet, and then as the major food source (Roberts, 2000). Finely ground, fresh seaweeds (Gracillaria and Lessonia sp.) are well accepted by both *H. iris* and *H. australis* and may be used as an intermediary and grow out food source (Brown pers. com. 1995).

In commercial hatchery and grow out operations juvenile abalone are provided with supplemental diatom until they can be weaned on to powdered artificial food diets when the juveniles are between 4 - 5 mm shell lengths (pers. obs. OceaNZ Blue Ltd., 2003).
1.7 Research Aims and Objectives

The research described in this thesis project had two main aims and objectives:

1. A key aim of this research was to examine the early life stages of *Haliotis australis* using scanning electron microscopy (SEM). The purpose of SEM analysis was to examine aspects of the morphology of the development of the gametes, larval and post larval stages and early juvenile stages of *H. australis* that were not able to be resolved using standard light microscopy techniques. The majority of detailed information of abalone larval and post larval development at the time of the research was in the form of drawings or photographs using light microscopy for other Haliotis species. The objective of the SEM analysis was to present a series of scanning electron photomicrographs documenting the development of the early life stages of *H. australis*. No previous SEM work had been published for this Haliotis species and the SEM photomicrographs presented in this thesis represent a new record of *H. australis* larval and early post-larval development from metamorphosis to 60-days post settlement.

2. The primary aim of the experimental part of the research was to spawn, raise and settle a batch of *H. australis* larvae on to four different types of settlement substrates that are typically used for the settlement of larval abalone in a commercial abalone hatchery and grow out facilities.

The settlement substrates tested were:

- An established diatom biofilm – ‘8 Day Ungrazed Biofilm’;
- An eight day old biofilm that had been pre-grazed by adult *H. australis* – ‘8 Day Grazed Biofilm’;
- A newly established diatom biofilm – ‘1 Day Biofilm’; and
- A control treatment containing no biofilm at settlement- ‘No Biofilm’.

An additional aim of the experiment was to test the effectiveness of a simple chemical treatment to induce settlement. The ‘chemical induction’ treatment involved the exposure of larvae to a ‘dip’ of the mammalian neurotransmitter γ-aminobutyric acid (GABA) prior to their introduction to the experimental system. The objective of the settlement experiments was to attempt to identify the best settlement substrate and a chemical induction technique to improve settlement of *H. australis* larvae within commercial abalone hatcheries.
Chapter 2 – Methods and Materials

2.0 Introduction

This chapter presents the materials and methods required to undertake the research including details of the location and source of the broodstock to be spawned, and the methods used to bring them into spawning condition prior to spawning induction. Methods of spawning induction and larval rearing and monitoring techniques used are presented in detail as are the techniques for preparation of samples for SEM. The research system setup, equipment and experimental design is described and discussed as are the methods used to prepare and maintain the experimental tank replicates. Data collection and statistical methods of analysis of results are also described.

2.1 Sources of Broodstock and Broodstock Conditioning

Brood stock used for the experiments were sourced from both wild stock and from hatchery reared animals. Five male and five female feral *H. australis* with shell lengths of approximately 90mm collected from natural sources were bought into spawning condition in the hatchery. The wild stock were collected from the Wairarapa coast by marine farmer Ralph Brown and conditioned at the Crystal Park Marine Farm facility (a small scale hatchery and grow-out facility located near Riversdale Beach on the Wairarapa east coast). These broodstock abalone were transported to the Rainbow Abalone Ltd. hatchery in New Plymouth in polystyrene boxes containing an ice pad that was covered by layers of seaweed (*lessonia variegata*). Transport by road took five hours and on arrival at the Rainbow Abalone Ltd facility, the abalone were placed into seawater maintained at ambient temperatures of approximately 15°C and kept in dark conditions in covered tanks to minimise stress post transport. The abalone were transported approximately two weeks before the commencement of the research.

Ten male and ten female *H. australis* were supplied for the research by Mr. Graeme Moss from the NIWA, Mahanga Bay hatchery in Wellington. These broodstock were second generation hatchery produced stock that were approximately 2.5 years old with a shell length approximately 65 mm. The *NIWA* broodstock abalone had been used in feed trials and had the characteristic shell color banding produced by the feeding experiments (Figure 1). The abalone were transported by courier in polystyrene containers lined with seaweed, then placed in dark storage tanks, with abundant feed and ambient seawater temperatures of 15°C. These abalone had been settled in Rainbow Abalone holding tanks for at least 6 months prior to the beginning of research.

All of the Yellowfoot Paua used in the research were conditioned prior to spawning by *ad libitum* feeding of a manufactured pellet feed product (sourced from South Africa) which was supplemented with fresh brown seaweed (*Lessonia variegata*). The abalone were kept in darkness at ambient temperatures of between 15-17°C throughout the conditioning period. Prior to spawning induction the
adult *H. australis* were selected and sexed before being placed into the spawning tubs containing separate sexes.

### 2.2 Hydrogen Peroxide Spawning Induction Method

*H. australis* were spawned using the hydrogen peroxide technique developed by Morse *et al.* (1977). The hydrogen peroxide spawning induction method (‘hydrogen peroxide method’) involved inducing spawning in conditioned adult abalone by exposing them to a weak solution of hydrogen peroxide that had been buffered by a sodium hydroxide solution in seawater.

#### 2.2.1 Spawning Solutions

##### 2.2.1.1 Hydrogen Peroxide (H$_2$O$_2$) Stock Solution

The hydrogen peroxide method requires a stock solution of hydrogen peroxide with a concentration of 100 volumes or 30%. The stock solution was kept in a fridge and in the dark as the hydrogen peroxide rapidly degrades on exposure to light. The stock solution deteriorates with time, and is required to be replaced every three to four months. The hydrogen peroxide stock solution was diluted with distilled water immediately before use to ensure maximum activity of the hydrogen peroxide (Tong *et al*., 1992).

##### 2.2.1.2 Sodium Hydroxide (NaOH) Stock Solution

A 1 M solution of NaOH was made by dissolving 40.0 g of sodium hydroxide pellets in distilled water and making up the solution to 1 litre. Due to the sodium hydroxide pellets being extremely caustic, a spatula and appropriate laboratory personal protective equipment (gloves, lab coat and eye protection) was used to hand spoon the pellets out for weighing and during mixing with the distilled water. The sodium hydroxide solution is able to be kept for later use without deterioration (Tong *et al*., 1992).

##### 2.2.1.3 Hydrogen Peroxide Spawning Techniques - Spawning Procedure

Thirteen female and fourteen male *Haliotis australis* were collected from broodstock holding tanks and placed in 10 liters of UV sterilised, 1 micron filtered sea water in plastic bins. The different sexes were segregated into separate bins. The abalone were treated with a solution of 6% hydrogen peroxide and 1M sodium hydroxide to induce spawning (Tong *et al*., 1992).

The spawning induction treatment has three key stages:

1) 50 ml of 6% H$_2$O$_2$ and 15 ml of 1M NaOH per 10 liters of water was added to the adult abalone for 1 hour.
2) The first solution was replaced with a half strength solution (25 ml of 6% H$_2$O$_2$ and 7.5 ml of 1M NaOH per 10 liters of UV sterilised, 1 micron filtered sea water) for 2 hours.

3) After 3 hours of immersion the chemical induction solutions, the bins containing the adult abalone were emptied and drained and filled with 1µm filtered, UV sterilised water at least three times. The spawning bins were then left undisturbed until spawning commenced. Throughout the spawning induction, black plastic covers were placed over the bins to restrict light. Spawned eggs were siphoned from the spawning tubs, washed, fertilised and placed into a specialised hatch tank (Tong et al., 1992)

2.3 Larval Rearing

Fertilised eggs of *Haliotis australis* were hatched and raised in a 500 litre continuous flow larval rearing system similar to that described by Tong *et al.* (1992). Seawater in which the larvae were cultured was filtered to 1 µm and UV sterilised. Swimming larvae were retained in the tank using a large ‘banjo’ sieve (mesh size 80 µm) at the outlet. Water temperature varied only slightly throughout the ten days of larval rearing from 15 - 16°C (ambient seawater temperatures).

The rearing tanks were covered during larval rearing period to help limit contamination from dust and airborne bacteria. Bacterial contamination in the larval cultures was detected using bacteriological plates and treated using three doses of the antibiotic streptomycin in a 24-hour period (Hahn, 1989d). Antibiotic treatment was necessary during critical periods of larval development process to avoid total mortality of larvae in the rearing system.

Development of the *H. australis* larvae was monitored using a light microscope, and the observation of completion of the formation of the larval shell marked the beginning of water changes in the rearing tanks. The complete formation of the larval shell was important as it enabled the larvae to be collected within a bucket sieve, washed and introduced back into rearing tanks that had been drained, cleaned and filled with sterilised seawater. Before the completion of the larval shell the larvae were too fragile to be handled and would have been killed if retained on a mesh screen during a water change. After completion of the larval shell, larval rearing tank water was changed three times daily. The water changes required siphoning the rearing tank water containing the developing larvae into an 80 µm mesh screen bucket sieve (Tong et al., 1992) that was sitting in a bucket containing isothermal water. The bucket sieve was gently removed from the bucket and *H. australis* larvae retained by the mesh screen within the bucket sieve were gently washed with 1µm filtered, UV sterilised seawater. The larvae were then rinsed back into a new rearing tank that had been cleaned, sterilised (with a wipe down with a weak bleach solution), dried, and filled with 1µm filtered, UV sterilised seawater.
2.3.1 Monitoring Larval Development and Competency - Radula Squash Technique

The ‘radula squash’ method described by Tong et al. (1992) was used to determine the stage of development of veliger larvae and their readiness for settlement. The radula squash technique involves dissolving the larval shell and squashing the larvae on to a glass slide so that the number of rows of chitonised teeth on the radula can be counted. When larvae have 10-15 rows of radula teeth present they may be sufficiently developed to ingest small diatoms (< 5 µm in size) and may be competent to settle (Tong et al., 1992).

Method used to prepare a radula squash:

1. Ethanol solutions of 70%, 90%, 95% were prepared;
2. A solution of 1 M KOH was prepared by adding 5.2 g of KOH to 100 ml of distilled water;
3. 30 – 40 larvae were collected in a small 80 µm mesh sieve;
4. The sieve was placed into 3 – 4 ml of seawater and 4 drops of concentrated HCl was added for four minutes. This treatment dissolved the larval shell but left the radula intact.
5. The larvae were then dehydrated using an alcohol series of 70%, 90%, 95% to 100% ethanol. The larvae were placed in each treatment for 2-3 minutes.
6. 10-12 larvae from the alcohol series were removed using a pipette and placed on a clean microscope slide to let the alcohol evaporate.
7. 2 drops of 1 M KOH were added to the slide. A glass cover slip was used to squash the larvae on to the slide. The number of rows of radula teeth were then counted under a light microscope.

2.3.2 Narcotisation of larvae - To Enable Analysis and Storage of Larvae

The velum of the free-swimming abalone larvae are very sensitive to chemical changes in the seawater and can be retracted into the larval shell for protection. When subject to chemical changes caused by chemical fixation the velum is retracted and cannot be seen when examined under the light or electron microscopes. To collect samples of larvae with their velum expanded, the larvae must be immobilised with a narcotising solution.

Table 1 details the components of the narcotising solutions required to immobilise and prevent contraction of the larval velum to allow examination of expanded velum structures under the light and scanning electron microscopes. (Stirling et al., 1984; Tong et al., 1992).
TABLE 1: SOLUTIONS REQUIRED FOR THE NARCOTISATION AND FIXATION OF MARINE INVERTEBRATE VELIGER LARVAE

<table>
<thead>
<tr>
<th>Calcium free seawater (g/L distilled water)</th>
<th>MgCl₂ (g/L distilled water)</th>
<th>Final narcotizing solution (g/5mL ethanol)</th>
<th>Fixative</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>33.5</td>
<td>Benzocaine 0.1</td>
<td>2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.6</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>12.3</td>
<td>0.1</td>
<td>(This solution is required to be made up fresh each day)</td>
</tr>
<tr>
<td>NaSO₄</td>
<td>3.9</td>
<td>Procaine 0.002</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.66</td>
<td>Add this to 45 ml of 50% seawater.</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.4</td>
<td>(This solution must be made up immediately before use)</td>
<td></td>
</tr>
</tbody>
</table>


Once narcotised, the veliger larvae were able to be placed in seawater in a watch glass and viewed and examined under a dissecting microscope without retraction of the velum occurring. The narcotising process has three stages that are described below.

**Stage I:** Seawater was gradually replaced with calcium free seawater (Table 1) over a period of 10 minutes, using a Pasteur pipette. The intention was to block synaptic transmission causing retraction of velum into larval shell. The treatment allowed the larvae to continue swimming, but appeared to block the normal retraction into shell caused by chemical or tactile stimulation.

**Stage II:** The calcium free seawater was gradually replaced by isotonic MgCl₂, and the larvae were left for approximately five minutes while the final narcotising solution was prepared. Isotonic MgCl₂ is a well-known narcotic for marine invertebrates (Stirling et al., 1984). The MgCl₂ slowed the swimming of the larvae so that, although the cilia were still beating, the larvae gathered at the bottom of the watch glass.

**Stage III:** The final narcotising solution contained benzocaine (ethyl-4-aminobenzoate) and procaine hydrochloride (Table 1). The MgCl₂ solution was gradually replaced with this mixture, which was added to a full watch glass to minimise turbulence. The veliger larvae were left for approximately 10
minutes until the cilia stopped beating. At any stage in the procedure, until the cilia stopped beating the narcotisation was able to be reversed by replacing the fluid surrounding the larvae with seawater.

**Fixation:** The narcotised veliger larvae were preserved for later examination using a process called fixation. A few drops of fixative (Table 1) is added to the narcotized sample. The fixative reacts with the final narcotising solution, this requires most of the fluid in the watch glass to be removed rapidly and replaced with fresh fixative. This procedure was repeated twice.

**Post Fixation:** After fixation, the fragile veliger larvae were rinsed several times in 0.1 M cacodylate buffer and post fixed in 1% OsO₄ (Osmium tetroxide) in 0.1 M cacodylate buffer.

Samples of veliger larvae were then stored in a refrigerator at the hatchery facility, until they were transported to Dunedin and prepared for SEM analysis.

### 2.4 Methods for Storage and Preparation of Samples for Scanning Electron Microscopy

During the research project samples of the *H. australis* gametes, larvae, post larvae and juveniles were taken from the spawning tubs, larval rearing and experimental tanks for examination under the light microscope. Samples at each development stage were also collected and fixed with 2.5% glutaraldehyde solution and post fixed with osmium tetroxide before being stored in rubber stoppered vials in a refrigerator. The samples were later prepared for examination under the SEM located at the School of Dentistry at the University of Otago. Preparation for examination of the samples using the SEM required the samples to be completely dry as the specimen chamber with the SEM operates using a high vacuum. All samples were post-fixed with osmium tetroxide, before being dehydrated using an alcohol series. Final preparation of the samples required critical point drying to remove all traces of water and ethanol before the samples were sputter coated with gold prior to storage and analysis in the SEM.

The process of preparation of samples for SEM analysis is described below:

**Fixation** – (to preserve and stabilise the soft body structures of the larvae and post larvae.)

Samples were immersed in a solution of 2.5% glutaraldehyde in a 0.2 M sodium cacodylate buffer followed by 3 rinses of 0.2 M sodium cacodylate buffer, pH 7.6.

**Post-fixation** – (use of osmium tetroxide embeds heavy metals directly into cell membranes which allowing scattering of electrons, and also stabilises proteins.) The samples are post fixed by immersing them in a 2 % osmium tetroxide solution in 0.1 M sodium cacodylate buffer (samples were able to be
set aside at this point without deterioration). Prior to dehydration each of the samples were subject to three rinses of 0.2 M sodium cacodylate buffer (to remove residual traces of osmium tetroxide).

**Dehydration series** – (The alcohol dehydration series replaces water in the samples with ethanol which helps to avoid shrinkage and collapse of soft body structures that may be caused by air drying.)

This involved:

- Samples were rinsed in a 50% ethanol solution;
- Samples were then rinsed in a 70% ethanol solution (samples can be set aside at this point without deterioration);
- Samples were then rinsed in a 90% ethanol solution; and
- Followed by two rinses in 100% ethanol.

**Critical Point Drying** – (where residual water and ethanol solvents are replaced by liquid CO₂.)

The dehydrated sample is then ready for drying in the Bio-Rad CPD 750 critical point dryer. The critical point drying process removes all traces of alcohol and water using a series of carbon dioxide rinses.

**Mounting and gold sputter coating**

After critical point drying the samples were mounted on aluminum electron microscope stubs, using double-sided carbon tape (carbon tape allows the transmission of electrons). The mounted and dried samples were then sputter coated with a fine layer of gold in a Polaron E5700 sputter coater. The sputter coating provided a surface that reflects the beam of electrons used to image the surface of the sample. The sputter coated samples were ready for analysis under the SEM and were able to be stored safely in a dehydration cabinet indefinitely.

**Scanning Electron Microscope Analysis**

Samples prepared for SEM analysis were examined using a Cambridge Stereoscan 350 Electron Microscope located at the University of Otago School of Dentistry with the assistance of an experienced SEM technician.

**SEM data capture and display**

Large format photo micrographs were taken during the examination of the samples, and the film processed and developed in a darkroom using standard photographic techniques. Video of the SEM
examination was also recorded direct onto a VCR from the electron microscope. Photomicrograph prints were later scanned into digital format and have been presented in the results section of this thesis.

2.4.1 Measurements Using SEM

Measurements of anatomical features of larvae and post larvae (e.g. shell length) were obtained using a measurement function on the SEM. The measurements of shell length that are recorded in the results section of this thesis were taken from the rear of the larval shell to the furthest point on the leading edge of the peristomal shell. The shell length measurements recorded using this technique were taken from a small random sample of SEM mounted larvae that were sourced from the combined sample of larvae and post larvae taken at a each sampling point during the experiment.

2.5 Experimental System - Factors Affecting Settlement

Figure 5 Shows the experimental system used in the settlement and survival experiments.

Figure 5: Photograph of the tiered experimental system used for settling and raising *Haliotis australis* larvae at Rainbow Abalone Ltd. hatchery in New Plymouth.
2.5.1 Experimental Settlement Tank & System Design

The experimental system used for larval settlement, post larval rearing, and grow out was a ‘flow through’ design using a single pass of filtered seawater into square plastic tanks (Figure 5). The aim of this design was to try to simulate the conditions found in standard 500 litre tanks, that were in use at Rainbow Abalone Ltd., on a smaller scale.

Forty plastic tanks were used in the experiment. The tanks were arranged on shelves in five levels with eight tanks on each level. Each tank had a working volume of 10 litres and was supplied with 1 µm filtered seawater at a flow rate into the tanks of approximately 1 litre/min. Water supply to the hatchery was supplied from the Rainbow Abalone Ltd. grow out system (a ring main system providing continuous flow of natural seawater that had been filtered through a nominal 50 µm diatomaceous earth filter).

Air was provided to each experimental tank from a 25 mm PVC pipe ring main that was located above each row of tanks. The air supply ring main was connected to the main hatchery air supply. A 5 mm plastic tube, tapped into to the ring main and was fitted with an aquarium air stone, was suspended into each experimental tank. Airflow to each experimental tank was regulated so that a maximum flow rate of air was achieved without creating excessive turbulence with the tanks.

Lighting to the experimental tanks was provided by florescent tubes (standard) mounted 300mm directly above the tanks in sealed plastic covers. The light covers were lined with strips of gaffer tape to reduce the light to achieve the desired light intensity. Light intensity during experiment was approximately 35–40 mE/m²/sec on a 12-h: 12-h light/dark cycle.

Wastewater from the experimental tanks flowed out of the experimental tanks via a 15 mm plastic pipe inserted in the front side (approximately 50mm from the top edge) of the tank, into a PVC guttering that was positioned along the leading edge of each shelf. The wastewater was directed through downpipes on to the hatchery floor and out into the main wastewater system of the hatchery facility.
2.5.2 Experimental System Maintenance and Sample Collection

Every 4 – 7 days a ‘drain and fill’ was conducted on each of the experimental tanks. The drain and fill required the tanks to be completely drained and gently rinsed with 1 µm filtered seawater to attempt to remove dead or dying larvae, non-attached diatoms, copepods and ciliate blooms from the tanks before they were refilled with fresh 1 µm filtered seawater. Debris residues were collected from each experimental tank during each drain and fill in to a large 80 µm screen mesh bucket sieve and then gently rinsed with fresh seawater before being transferred to a smaller 80 µm sieve for examination under a dissecting microscope. Samples were taken from the residue for further examination under the light and dissecting microscopes and a portion of the sample was also fixed and stored for later SEM analysis. The setup for the drain and fill operations is shown in Figure 6.

Figure 6: Set up for experimental tank maintenance and sample collection - 1st 'Drain and fill'.

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2.6 Experimental Design - Settlement Factors

Experiments were conducted to determine factors affecting the success of settlement in *Haliotis australis*. The settlement experimental design had two parts;

1. **Settlement Factors** - Aim - To test the effectiveness of different ages of diatom surfaces commonly used in abalone hatcheries for settling larval abalone and on the settlement of *H. australis*.

2. **GABA Treatment** - Aim - To test the effectiveness of a known settlement inducer γ-aminobutyric acid (GABA) (Morse et al. 1979) to induce *H. australis* larvae to settle.

The 40 experimental tanks were divided into four settlement factors with 10 replicates for each of the four factors tested. Of the 10 replicates for each settlement factor tested, half of the replicates (five replicates) were treated with a 1 mM GABA solution.

Each of the replicates was assigned a tank number and were randomly assigned to a position within the framework of the experimental setup, to limit bias.

### 2.6.1 Settlement Factors

The four experimental settlement factors tested were:

1. **8 Day Ungrazed Biofilm**; Settlement on an established 8 day old diatom biofilm.
2. **8 Day Grazed Biofilm**; Settlement on an established 8 day old diatom biofilm pre-grazed by conspecific adult abalone (*H. australis*).
3. **1 Day Biofilm**; Settlement on a 1 day old diatom biofilm.
4. **No Biofilm**; Settlement on surfaces with no diatom biofilm (control treatment).

### 2.6.2 GABA Treatment

The GABA treatment consisted of immersing competent larvae of *H. australis* contained within an 80 µm mesh bucket sieve, into a bucket containing a solution of GABA and seawater made from dissolving 1 g of γ-aminobutyric acid in 10 liters of 1µm filtered, UV sterilised seawater. The larvae were exposed to the GABA solution for thirty minutes prior to their introduction into the settlement tanks.

### 2.6.3 Preparation of Experimental Tank Diatom Biofilms

Diatoms used for seeding the experimental systems were cultured in the hatchery’s bulk diatom culture system. The bulk culture system consisted of a series of approximately 50 meter long lengths of clear flexible plastic pipe (approximately 25 mm in diameter) that had been seeded with a diatom slurry from
a previous diatom harvest. The bulk culture pipes were seeded with a solution containing several liters of diatom slurry fertilised using a proprietary liquid fertiliser (e.g. Yates ‘Thrive”) and a silicate solution. The seeded pipes were left for 24 hours before a continuous water flow from the hatchery system was restored through the pipe. The attached benthic diatoms were grown on the internal surfaces of the clear tubing under continuous lighting from natural and fluorescent sources. Diatoms were harvested into a bucket by forcing a ‘pig’ (plugs of a nylon scourer) through the culture pipe under water pressure from the hatchery’s seawater ring main.

Diatom biofilms used in the experimental tanks were prepared by seeding the tanks with a mixed diatom slurry. Preparation of the diatom ‘seed’ slurry required the raw diatom slurry harvested from the bulk culture system to be passed through a 5µm filter to remove unwanted large diatom species and retain the small (<5 µm) benthic diatoms that would be able to be ingested by the settling *H. australis* larvae and post larvae.

15 ml of the seed diatom slurry was added to each experimental tank, along with 1 ml of a Guillard F/2 nutrient solution (Guillard, 1975) and 12 ml of a 15 g/l d H2O silicate stock solution (silicate is important for development of diatom skeletons). Water flow to each experimental tank was then stopped for 24 hours to prevent diatoms being lost from the tanks and to provide an opportunity for the diatoms to settle and attach to the experimental tank surfaces. To collect samples to analyse the biofilm using SEM several glass cover slips were placed onto the bottoms of the ‘8 Day Ungrazed Biofilm’ tanks prior to seeding of the diatoms into the tank and were removed and fixed and stored for later SEM analysis.

‘8 Day Ungrazed Biofilm’ and ‘8 Day Grazed Biofilm’ experimental tanks were seeded with diatoms eight days prior to the introduction of competent *Haliotis australis* larvae into the tank. Pre-grazing of algal biofilms to produce conspecific mucus trails within the ‘8 Day Grazed Biofilm’ experimental tank replicates was achieved by introducing two adult *H. australis* into each of the tanks for 48 hours prior to introduction of competent larvae. ‘1 Day Biofilm’ tanks were cleaned and inoculated with diatoms and nutrient solution approximately 24 hours before the introduction of competent larvae. The ‘No Diatom Biofilm’ tanks replicates were cleaned and refilled with filtered seawater immediately prior to introduction of competent larvae to the experimental tanks. Competent *H. australis* larvae were introduced into all of the experimental tanks within an approximately two hour period on Day 0 of the experiment.
2.7 Data Collection

The initial experimental design sought to investigate settlement success by counting swimming larvae and larval mortalities collected from each tank at the first drain and fill maintenance. This was abandoned due to the large scale of the experiment, the large numbers of mortalities occurring within each tank and the difficulty of counting dead and swimming larvae using the equipment available at hatchery.

The experiment was initially established with a plan to collect settlement data from within each of the experimental tank replicates by using five plastic ‘bio-chips’ that had been attached to Velcro dots randomly positioned within each of the experimental tanks. The bio-chips were made from the same plastic as the tank and each bio-chip had dimensions of approximately 20 mm x 20mm. Their purpose was to provide a representative (removable) surfaces within each tank which could be removed for examination under the dissecting microscope to undertake counts of live animals. The collection of data to infer settlement success using the ‘bio chip’ method was difficult and problematic for the following reasons: the large scale of the experiment; the limited time to undertake observations; difficulties in handling the small plastic bio-chips; and difficulties in observing and obtaining counts of the (microscopic) larvae that had settled onto the bio-chips. This method of data collection was subsequently abandoned early in the experiment due primarily due to handling difficulties and the potential for cross contamination of experimental tanks. No larvae that settled and attached to the bio-chips survived key periods of mass mortality in the first 30 days of the experiment.

Instead settlement data used to infer settlement success for each treatment was collected by measuring survival within each experimental replicate at 33-days post settlement (43 days post fertilisation). Settlement data was obtained by undertaking a full count of individual juvenile Haliotis australis in each experimental tank at 33-days post settlement and was the time selected to begin counts to measure settlement and survival for the following reasons:

- Periods of high larval and post larval mortality had passed (high mortality was observed at 1-4 days post settlement and at 17-24 days post settlement);
- Development of the post larvae was at a stage just prior to the formation of the first respiratory pore; and
- Post larval H. australis were approximately 1mm in length and were able to be easily observed inside tanks with the naked eye.

Full counts of the surviving H. australis post larvae and juveniles in each replicate tank were taken at each drain and fill event from 33-days post settlement to 60-days post settlement.
2.8 Data Analysis - Statistical Analysis

Differences in survivorship of *H. australis* between the diatom film treatments (‘8 Day Biofilm’, ‘Grazed 8 Day Biofilm’, ‘1 Day Biofilm’ and ‘No Biofilm’ and the GABA / no GABA treatments, were examined with a two factor analysis of variance (ANOVA), using Minitab 16 (Minitab Inc., Pennsylvania, USA).

The Anderson-Darling test for normality and Bartlett’s test for equal variance were conducted prior to ANOVA to ensure that the data complied with the assumptions of normality and homogeneity of variance. Transformations of the data were performed to ensure that these assumptions were met. The results of Anderson-Darling test for normality and Bartlett’s test for equal variance are presented as Table 2 in the results section of this thesis.

The raw data was transformed using a Log\textsubscript{10} transformation so that it was normally distributed prior to the analysis. When significant differences were detected (p-value < 0.05), multiple comparisons between site means were conducted using Tukey’s method (equal number of observations per treatment).
Chapter 3 - Results

3.1 Introduction – Overview of Results

The following results chapter is presented in two parts.

Part one of this chapter presents the results of the growth and development investigations into *H. australis* and includes the following:

- A record of the ambient seawater temperatures that were obtained from the Rainbow Abalone Ltd seawater intake over the period of the experiment.
- The results of measurements of the shell lengths of post larval *Haliotis australis* from samples taken at each drain and fill sample collection (occurring at 4-8 day intervals) during the experiment.
- Descriptions of the detailed SEM photomicrographs of larval and post larval stages of *Haliotis australis* from samples collected during the spawning and larval rearing period, and also during the 60-day post settlement experimental period. Observations recorded during the same period are presented in the results alongside the relevant SEM photomicrographs. Aspects of the general physiology of *Haliotis australis* have been described in the results section using the photomicrographs of 60-day post settlement *H. australis* juveniles.

Part two of this chapter presents the results of the settlement experiment undertaken to test the factors affecting settlement of *H. australis*. For each of the four experimental settlement treatments (8 Day Ungrazed Biofilm, 8 Day Grazed Biofilm, 1 Day Biofilm and No Biofilm) the following results are presented:

- The results of the survival data from the tank counts undertaken at 33, 39, 45, 52 and 60-days post settlement are presented in graphical form to enable visual comparison of the data to be made;
- Presentation of the results of statistical analysis of the 33 day post settlement data using two factor Anova, and Tukey tests to determine if there were any significant differences between the settlement treatments or the GABA Treatment factors being tested;
- A brief analysis of survival from 33-days post settlement to 60-days post settlement is undertaken for each experimental biofilm treatments; and
- Observations for each of the diatom biofilm treatments that were recorded during the experiment.
3.2 Results Part 1 – Observations of Spawning, Larval and Post Larval Growth and Development to 60-Days Post Settlement.

3.2.1 Ambient Seawater Temperature During the Experiment

The induction of spawning of the *H. australis* was initiated in mid-November 1995 at the Rainbow Abalone Ltd. hatchery facility when ambient seawater temperatures increased to approximately 15°C had been maintained for several days.

The hatchery facility did not have the means to control water temperature, so spawning induction was timed to correspond with steady ambient seawater temperatures to ensure that larval rearing could be undertaken at approximately 15°C for the duration of the larval rearing period. Figure 7 shows that the ambient seawater temperature (as measured at the hatchery intake) supplied to the experimental systems rose over 6 °C in the approximately nine weeks of the experiment (from November 1995 to January 1996) and ranged from a temperature of 14.1 °C recorded on day 14 post fertilisation (4-days post settlement) of the experiment to a temperature of 21.9 °C recorded on day 66 post fertilisation (56-days post settlement).

![Figure 7: Daily ambient seawater temperatures measured during the period of the settlement experiments from 15 November 1995 to 26 January 1996 as measured at the hatchery seawater intake.](image)

Ambient seawater temperatures continued to increase following the conclusion of the experiment peaking at 23°C in early February 1996. These sustained elevated temperatures are likely to have contributed to the significant mortality of the 2185 remaining *Haliotis australis* juveniles surviving at the end of the 60 day settlement experiment. One month following the end of the experimental period only one juvenile *H. australis* (with a shell length of approximately 10mm) had survived (Watts pers. comm. 1996).
3.2.2 Shell Growth of Post Larvae

A small sample of post larva shell measurements were taken using the measurement function on the SEM. An average daily growth of peristomal shell growth was calculated from the data and the mean shell lengths of post larvae were measured and plotted against time (Figure 8) to give an indication of the trends in shell growth with the age of post larvae. The trend in growth rate appears to fit an exponential curve, however care should be taken in relying on the reported rates of shell growth as measured. These shell growth rates can only be interpreted as an indicator of the actual growth rates occurring at a particular period due to the low number of individuals measured, and the likelihood that the increasing ambient seawater temperature throughout the experiment had a positive effect on shell deposition rates.

Figure 8: Mean shell length measurements and SE of *H. australis* post larvae throughout the experiment.

Days post settlement = Days post fertilisation +10 days; Error bars = SE; Mean± one standard error. n=6 from 4 to 45 days post settlement; n=4 at 52 days post settlement; and n=3 at 60 days post settlement.

Daily growth rates appeared to slow during periods of high mortality, which occurred between 12-14 days post settlement and again at 17-21 days post settlement. At 14-days post settlement the post larval shell had developed a symmetrical (circular) shell form and had a shell length of approximately 500 µm. Observation of post larvae under the light microscope showed that the post larvae appeared to be less active than in earlier post larval forms and had difficulty moving through the thickening diatom biofilm. The post larvae also appeared to have difficulty lifting the shell edge above the diatom biofilm. It may be that a potential cause of the mortality during this period was due to starvation as a result of
inability of post larvae to access sufficient food resources required to maintain the rapid shell growth and physiological development.

At 17 – 21 days post settlement the shell growth form was an asymmetrical oblong (ear shaped) form and SEM imaging shows that the peristomal shell was beginning to overlap onto the larval shell for the first time. The additional shell secretion required to overlap the shell may have required additional energy resources and this may have contributed to mortality. Observations of diatom biofilm corresponding to the same period within some of the ‘8 Day Grazed Biofilm’ and ‘8 Day Ungrazed Biofilm’ replicate tanks showed patchy diatom growth and large areas of tank surfaces that appeared to be completely grazed by post larvae. At this stage high mortality of post larvae was observed in all experimental tanks with mortalities particularly apparent in the ‘8 Day Ungrazed Biofilm’ and ‘8 Day Grazed Biofilm’ replicates.

The low rate of shell growth (approximately 11µm/day) and high periods of mortality between 17-21 days post settlement are likely to have occurred as a result of starvation arising from lack of suitable or available diatoms for ingestion by the developing post larvae within experimental tanks. These suspicions were confirmed by comments provided on the SEM images (Figure 25) from this period of larval development by Dr. Roberts (pers. com., 2000), who indicated that the post larvae appeared to be severely starved. Overgrazing problems were not observed in ‘1 Day Biofilm’ or ‘No Biofilm’ treatment replicates.

In response to the overgrazing, additional diatom slurry (in similar amounts to that used initially to inoculate the tanks) was added to the experimental tanks during the period between 19-21 days post settlement, and this was subsequently repeated at each drain and fill, to supplement the food supply for the developing post larvae. Supplemental feeding appeared to be important particularly within heavily grazed ‘8 Day Grazed Biofilm’ and ‘8 Day Ungrazed Biofilm’ tanks. The apparent improved health and growth of the post larvae following the addition of supplemental diatom in experimental tanks between 21 - 28 days post settlement can be seen in an increase in average shell length of approximately 68 µm daily (a fivefold increase from the previous period). There was a slight decrease in shell length growth rate between 28-33 days post settlement (approximately 45 µm increase daily) however this corresponds to a period of falling ambient seawater temperatures (19.4°C to 18.4°C).

Growth rate of the larval shell appeared to slow again between 33-41 days post settlement when the first respiratory pore was being formed (39 µm daily increase in shell length). Juvenile shell growth rates increased to an average rate of approximately 85 µm per day over the four days following the formation of the first respiratory pore, and then sustained shell growth rates of approximately 95 µm per day from 45-52 days post settlement were observed when ambient seawater temperatures were steady at around 20.3°C. A rapid increase in, and fluctuation of, ambient seawater temperatures from
20.3°C to 21.4°C (peaking at 21.9) over the eight days between 52-60 days post settlement may have contributed to a decrease in shell growth rate to approximately 81 µm per day.

3.2.3 Observations of *Haliotis australis* Spawning - Male.

The gonad of the male *H. australis* was a creamy yellow colour. When ready to spawn the gonad was easily seen as a large cream pouch located near the shell spire. Observation of the gonad was achieved by gently lifting back the epipodium and mantle flap close to the shell coil with a blunt knife.

The male abalone showed no noticeable behaviour during the exposure to the hydrogen peroxide spawning induction treatment. On rinsing the male brood stock three times with fresh seawater following exposure some individuals began spawning immediately. The male *H. australis* were observed to raise their shell and then contract the shell down onto the foot. With each contraction of the shell, jets of milky fluid (spermatozoa) were observed to exit the respiratory pores of the abalone. The water in the male spawning tubs quickly became opaque in colour and clouded with spermatozoa (Figures 9 and 10), and within minutes no further observations on behaviour were able to be made. However observations of behaviour obtained two hours later during the rinsing and refilling the spawning tubs with fresh seawater showed that spawning contractions were still occurring.

![Figure 9: *Haliotis australis*; Mass of spermatozoa.](image)

(s) Spermatozoa.
Examination of the gametes under the light microscope showed the spermatozoa to be highly active and swimming strongly. Observations of the spermatozoa under the SEM clearly show the physiological features of the male gametes of *Haliotis australis*. The mature sperm has three parts; the head; the middle segment; and the axial filament. The head is an elongated cone containing the nucleus and is surrounded by the acrosome. The middle segment is 1 μm in width, 8 μm in length, cylindrical in shape and slightly longer than the head. At the base of the middle segment is a rosette of five mitochondria bundles. The axial filament propels the sperm and is approximately 50 μm long. The close up image of individual spermatozoa in Figure 10 clearly shows the parts of the spermatozoa; axial filament (fl), mitochondria bundles (mb), mid segment (ms) and acrosome (a).

**3.2.4 Observations of *Haliotis australis* Spawning - Female.**

The gonads of the female *Haliotis australis* were observed to be a rich purple color. The females began exhibiting spawning behaviour approximately 1-2 hours following the removal of the spawning induction solution from the spawning tubs and rinsing with fresh seawater.
The typical spawning behaviour observed as being exhibited by *H. australis* females was the raising of the anterior portion of the shell, flaring and extension the epipodium, with this position held ridged in a state of tension. This behaviour was followed by a rapid contraction the epipodium two or three times before forcefully clamping down or sinking down on to the foot. At the same time as the contraction of the shell down on to the foot, streams of purple eggs were observed being released through the 2nd and 3rd respiratory pores, carried by the water currents generated by the contraction. The cycle of behaviour occurred over a five to ten minute period and was repeated. The eggs were heavier than seawater and quickly settled to the bottom of the spawning tubs.

![Eggs](image)

**Figure 11: Haliotis australis; a cluster of unfertilised eggs.**

*(e) H. australis egg*

Observation of the eggs under the light microscope showed the eggs had a dark purple colour and were approximately 180 μm in diameter. Samples of the eggs were collected and stored in glutaraldehyde for later preparation and examination under the SEM. Figure 11 shows a SEM photomicrograph unfertilised eggs.

The female *H. australis* broodstock that were sourced from the Mahanga Bay hatchery ejected fine streams of eggs that separated and dispersed quickly. These female broodstock were approximately 65mm long and approximately two years old. The larger wild stock sourced from the Wairarapa coast (approximately five years old and 90mm in length) were observed to eject large clumps of eggs that did not separate easily when disturbed by a fine stream of fresh seawater.
The eggs were siphoned out of the spawning tubs using a five millimeter diameter flexible tube at regular intervals, and were gently rinsed for approximately five minutes through a 250 μm sieve and collected in an 80 μm mesh screen bucket sieve (submerged in fresh 1 μm filtered seawater) to remove debris and damaged eggs. The spawning event lasted approximately three hours following the initiation of spawning and produced an estimated yield of approximately 1.5 million eggs.

Gently stimulating the spawning *H. australis* with a stream of fresh seawater was observed to cause the abalone to perform additional rapid contractions during spawning, which appeared to speed and enhance the completeness of the spawning event. This technique had been identified as being successful in enhancing spawning activity in *H. iris* (Pers. comm. R. Brown & E. Watts, 1995). Raising water temperature slowly over a period of a week prior to a spawning event will also help make spawning events more successful (Pers. com. E. Watts 1995). The slow increase in temperature of the broodstock conditioning tanks prior to induction of spawning was considered a beneficial practice to assist the final development and maturation of the gametes before spawning.

### 3.2.5 Fertilisation of *Haliotis australis* Gametes

At the end of the three hour spawning period, 100 ml of freshly spawned spermatozoa was gently mixed into the rinsed eggs that were in a bucket and contained within the 80 μm mesh screen bucket sieve. The eggs and spermatozoa solution was left for five minutes to allow fertilisation to occur. Freshly spawned spermatozoa was used for fertilisation because the spermatozoa only remains viable for a short time. A diluted solution of spermatozoa was used to ensure that polyspermy (fertilisation of egg by several sperm) did not occur. The concentration of the spermatozoa solution was measured by eye and had an opaque colour, similar to that of a solution of diluted milk.

The fertilised eggs were the placed into a hatch tank and provided with a constant supply of 1 μm filtered seawater at an ambient temperature of 15°C. Observations of the early development were conducted over the few hours following fertilisation by examination under a light microscope.

Major observations post fertilisation included:

- the ejection of the polar body after approximately 15 minutes post fertilisation
- 1st cell division occurred 1.5 hours post fertilisation.
- The eggs hatched at 20 hours post fertilisation at ambient temperatures of 15°C.
3.2.6 Larval Rearing

3.2.6.1 Day 2 Post Fertilisation (16th November 1995)

After spawning the fertilised eggs of *Haliotis australis* were placed in to a hatch tank based on design of Tong *et al.* (1992). After 17 hours of development at 15°C the gastrula of *Haliotis australis* had formed and the cilia that form the prototrochal girdle were visible. At this stage the embryo can be classified as an early trochophore. The embryo continued development within the egg membrane and cilia were observed to grow in a band around the anterior end of the early trochophore, this band is known as the prototrochal girdle (pg). At 18-19 hours post fertilisation many of the *H. australis* trochophore were beating their prototrochal cilia and were beginning to rotate within the egg membrane. Unfertilised eggs were be easily detected as this stage by their lack of cell differentiation and inactivity.

![Figure 12: *Haliotis australis*; Trochophore larvae at 23 hours development at 15°C.
(pg) prototrochal girdle; (sf) shell field.](image)

Figure 12 shows the cilia that form the prototroch girdle and the early stages of mineralisation of the larval shell can be observed in the oval shield field (sf). When the prototrochal girdle was fully formed the embryo is considered to be a trochophore.
The cilia of the prototrochal girdle cause the trophophore to spin within the egg casing. At 20 hours of development at 15°C, the spinning trophophore burst the egg membrane and hatch out occurred. The trophophore larvae (Figure 12) swam to the surface of the hatch tank and were carried by the water current within the rearing system to the hatch tank out-flow where it overflowed into the larval rearing tanks. Larval rearing tanks used in the Rainbow Abalone Ltd hatchery were based on a similar design as those described by Tong et al. (1992).

![Image of early veliger larvae](image.jpg)

**Figure 13: Haliotis australis; View of apical region and velum, 72 hours post fertilisation.**

(v) Velum; (pc) protoconch; (at) apical tuft.

The trophophore maintained their position in the water column using the rhythmic beating of the band of prototrochal cilia. Secretion of the larval shell or protoconch (pc), begins from within an area on the posterior of the trophophore known as the shield field (Figure 12). Development of the trophophore continued over the next 48 hours into the early pre-torsion veliger larval stage. As development of the trophophore progressed increased cell differentiation occurred posteriorly and this development was enclosed by the protoconch (Crofts, 1937). The cilia of the prototrochal girdle developed into the early velum (v). The apical tufts (at) on the anterior of the early veliger are able to be observed as the cilia located in the center of the velum in Figure 13.
Within 72 hours post fertilisation the trochophore had developed into a pre-torsion veliger larva. The apical region had flattened and the prototrochal girdle of the trochophore had developed into the velum, which continued to beat and maintained the developing larvae in the water column. The larval shell was not yet fully formed and torsion had not yet occurred (Figure 13 and Figure 14).

![Image of early veliger larva](image)

**Figure 14: Haliotis australis; Pre-torsion veliger larvae 72 hours post fertilisation.**

(v) velum; (pc) protoconch.

Over the next 24 - 48 hours the early veliger larva underwent torsion, where the protruding foot mass and viscera twists 180° from its original position, and the operculum was observed to have formed. The larvae was at the stage where the foot had begun to differentiate and the larval shell was still soft and fragile. Due to the fragile nature of the developing larvae from days 1-5 post fertilisation, the handling or disturbance of the larvae by culturists is not recommended during this period.

### 3.2.6.2 Day 5 Post Fertilisation (20th November 1995)

Bacterial levels were high in the hatchery tanks as indicated by bacteriological plate bacterial counts from water samples taken from the larval rearing tanks. Dead and dying larvae were observed to have settled on to the bottom of the larval rearing tanks and were a potential source of contamination if left in tanks. Actively swimming and healthy larvae were isolated by removing the banjo sieve over the..
outflow (Tong et al., 1992) and the larvae were transferred into a clean bucket containing an 80 µm bucket sieve. The transfer of larvae will almost certainly transfer the bacteria causing the contamination problems. The veliger larvae isolated in the separate container using a submerged sieve were then treated with antibiotics while the rearing tanks were drained, cleaned with a weak bleach solution, dried and filled again with 1µm filtered UV sterilised seawater ready for the return of the larvae. A full course of three treatments with the antibiotic streptomycin was conducted and combined with a change of water in the larval rearing tanks during the ten day larval rearing period.

Veliger larval counts were undertaken at this stage when the larvae were concentrated for antibiotic treatment. Estimates of the total number of viable larvae within the rearing tanks were obtained by counting the larvae in 1 ml taken from 10 liters of culture water containing all of the larvae. The counts indicated that the rearing tanks contained approximately 330,000 healthy swimming larvae.

A radula squash was performed at day 5 post fertilisation to count the rows of chitonised radula teeth. The procedure was unsuccessful at this time, with the radula unable to be located.

3.2.6.3 Day 6 Post Fertilisation (21st November 1995)

Figure 15 shows a side on view of a Haliotis australis veliger larvae a fully formed larval shell (ls), partially retracted foot (f) and collapsed velum (v) (note the absence of the operculum in this view).

Examination of the larvae under the light microscope showed three tubules formed on the developing cephalic tentacles. Radula squash of the larvae from the tanks at day 6 post fertilisation showed that 4 - 5 rows of radula teeth were visible. The growth of the radula teeth became the most important indicator of development from this time. The larval shell was fully formed and robust. The velum was fully formed and exhibited a characteristic beating motion enabling the larvae to maintain their position near the surface of the rearing tanks. The larvae were positively phototactic and concentrated near the surface when a light source was placed over the tanks in a darkened room. Samples of larvae were narcotised successfully and the protracted velum was observed under the light microscope. Samples of narcotised larvae were taken and fixed with 2.5% glutaraldehyde, however the rigors of storage and preparation for electron microscopy has caused the collapse of the veliger’s swimming apparatus (velum) and likely caused the loss of the operculum of the veliger larva shown in Figure 15.

3.2.6.4 Day 7 Post Fertilisation (22nd November 1995)

Radula squash of larvae early in the day 7 post fertilisation showed six rows of radula teeth had developed, with a 7th row developing. Late in the day radula squash analysis showed seven rows of marginal teeth had developed and the lateral teeth were beginning to form. Ambient water temperature had increased to 16.5°C.
3.2.6.4 Day 8 Post Fertilisation (23rd November 1995)

Early in the day 8 post fertilisation the veliger larvae had eight rows of marginal radula teeth, and by the end of day 8 the larvae had nine rows of teeth with the 10th row developing.

3.2.6.5 Day 9 Post Fertilisation (24th November 1995)

For the purposes of this research (and from recommendations of NIWA scientist Dr. Len Tong), larvae with ten rows of marginal teeth were considered ‘competent’ and ready for settlement into the experimental system. By midmorning on day 9 post fertilisation at 15-16°C, all larvae sampled showed that at least nine rows of radula teeth had developed.

Some of the veliger larvae were observed to exhibit the crawling behavior which precedes settlement and metamorphosis of the larvae from a free-swimming, non-feeding larvae to a feeding benthic post larva. When disturbed these crawling veliger larvae were able to return to the water column and continueswimming using their velum. The veliger larvae on day 9 post fertilisation had visible cephalic tentacles with three to four tubercles present on each of the cephalic tentacles.
The key features of the crawling veliger larva are visible in Figure 16, with the features to note being the larval shell (ls), collapsed velum (v), cephalic tentacles (t), an operculum (op) and well differentiated foot (f). There are four sensory tubercles visible as protrusions on the cephalic tentacles present on each of the cephalic tentacles.

![Crawling veliger larva with labeled features](image)

**Figure 16: Haliotis australis; a competent 9-day-old larva, ready for settlement.**

(v) velum; (f) foot; (ls) larval shell; (ct) cephalic tentacle; (m) mouth; (op) operculum.

### 3.2.7 Observations of Post-larval Development to 60-days Post Settlement

For the purposes of this research the start of the experiment testing settlement on diatom films of different ages and GABA treatment as a settlement inducer was determined to be when the competent larvae were introduced into the prepared experimental tanks. This occurred on 25 November 1995 (day one post settlement) when the *H. australis* larvae were deemed to be competent to settle and were introduced into the experimental tanks. The water and air supply were shut off for the first 24 hours to encourage settlement of the larvae. Swimming veliger larvae were retained within the experimental replicate tanks by 80 µm mesh screen banjo sieves fixed to the outflow pipes inside each tank. Water flow and air supply to the experimental tanks was restored after 24 hours.
3.2.7.1 Observations 4-Days Post Settlement

Unsettled and swimming larvae were removed from the experimental systems at the first drain and fill on 4-days post settlement (day 14 post fertilisation). The veliger larvae were now 14 days old and had 14 rows of radula teeth. The larval shell was measured at approximately 270 μm in length.

![Image of 4-day postlarva](image)

**Figure 17: Haliotis australis; Lateral view of a 4-day post settlement larva.**

(ls) larval shell; (ps) peristomal shell.

Examination of 4-day post settlement larva (Figure 17 and Figure 18) elucidates the two key features of the newly settled developing post larvae as being the larval shell (ls) and the new growth of peristomal shell (ps).

The larval shell is stippled and textured with the peristomal shell is growing in a clockwise spiral with distinct grooves or banding. The interface between the larval shell and the peristomal shell can be seen clearly and growth follows the contour of the larval shell. The leading edge of the shell that develops along the interface of the larval shell has reduced ridging in the early peristomal shell development.
Mean shell length (length of larval shell and peristomal shell) at 4-days post settlement was measured at 343 μm (Figure 8) with an average daily increase in shell length of 18.3 μm at ambient seawater temperatures of 15.4°C.

Figure 18: *Haliotis australis*; dorsal view of a 4-day old post larva with ridged peristomal shell growth.

(ls) larval shell; (ps) peristomal shell.

Growth of the peristomal shell indicated that metamorphosis had been initiated. The dorsal view of the 4-day post settlement larva (Figure 18) shows the characteristic early asymmetric growth form described by Leighton (1974). This view gives a good indication of the position a developing larva has on the substrate during the early stages of growth. The position of the larval shell is vertically oriented and a feature of peristomal shell growth is the strong differentiation from right to left (when viewing the larval shell from a posterior aspect). The left side of the shell has sharply defined ridges compared with the right side, which is smooth and rounded.

Observations under the light microscope showed the heart had developed and was observed beating in 4-day post settlement larvae.
3.2.7.2 Observations 8-Days Post Settlement

Advanced peristomal shell growth is visible in 8-day post settlement post larva (Figure 19), this stage of development corresponds to the mid-asymmetric growth form described by Leighton (1974). The feature to note in Figure 19 is the contoured growing edge of the peristomal shell over lapping the larval shell, and the position of the larval shell relative to the peristomal shell. The larval shell has rotated with the spiral growth of the peristomal shell and is now aligned almost in a horizontal position (when compared with the 4-day post settlement post larva shown in Figure 18). This spiral shell growth is a feature of univalve gastropods (Barnes 1986).

Figure 19: Haliotis australis; Lateral view of 8-day old post-larva.

(ls) larval shell; (ps) peristomal shell.
3.2.7.3 Observations 12-Days Post Settlement

Two views of post larvae sampled at 12-days post settlement are shown in Figure 20. The ventral view of the larva at the center gives a good view of the ridged margin of the peristomal shell. The anatomical features of the post-larva are difficult to separate in the view of this post-larva. The operculum is still present in some individuals at 12-days post settlement. The dorsal view of the post larva in Figure 20 (at top left of the photomicrograph) shows the growth of the peristomal shell giving the larvae characteristic mid-asymmetrical, oblong shape. Mean shell length of *Haliotis australis* post larvae at 12-days post settlement (day 22 post fertilisation) was 439 µm (Figure 8). There was an average daily increase in shell length of 12.0 µm at ambient seawater temperatures of 16°C since 4-days post settlement. The other organism present in Figure 20 is a male copepod of the crustacean class Copepoda.

![Figure 20: Haliotis australis; two views of 12-day old post-larvae and a copepod.](image)

(f) foot; (ls) larval shell; (ps) peristomal shell; (ct) cephalic tentacle; (ma) mantle; and (op) operculum.
Figure 21: *Haliotis australis*; Ventral view of a 12-day old post-larva.

(f) foot; (ls) larval shell; (ps) peristomal shell; (ct) cephalic tentacle; (ma) mantle; (rs) radula sheath; (op) operculum.

Figure 21 provides an excellent view of a 12-day post settlement larva showing the ‘snout’ or radula sheath (rs) (which contains the mouth and radula) flanked by the two cephalic tentacles. Each of the cephalic tentacles has seven or eight buds or sensory tubercles present at this stage.

The outer membrane (periostracum) of the larval shell in Figure 21 shows deterioration (caused by the chemicals used to fix the larvae for storage) and is peeling away revealing the smooth calcified larval shell underneath. Benthic diatoms are visible attached to the underside of the peristomal shell.

The first of the epipodial tentacles had formed and was visible under the light microscope. The operculum (op) was visible in some post larva at 12-days post settlement, indicating that post-larvae of *H. australis* may retain the operculum for some time post settlement.

### 3.2.7.4 Observations 14-Days Post Settlement

At 14-days post settlement the post larvae were showing rapid peristomal shell growth and a more symmetrical shell form. Figure 22 shows the symmetrical growth form of the peristomal shell in a 14-day post settlement larva as described by Leighton (1974). When larvae reached this stage of symmetrical shell growth, high mortalities were observed in all experimental tanks.
At 14-days post settlement the peristomal shell was observed to be much larger than the developing foot in many specimens and it was observed that many of the post larva had difficulty moving amongst the rapidly growing diatom biofilm in the settlement tanks. Clumps of diatoms (d) from the settlement tank can be seen by the post larva shell edge and a large diatom (approximately 110 µm in length) is visible at the bottom left of the micrograph in Figure 22.

In Figure 23 the radula sheath (rs) or ‘snout’ of the 14-day post settlement post larva is clearly visible and the cephalic tentacles have up to ten sensory tubercles each. The foot (f) has elongated cilia at its forward margin. The post larvae are able to secrete globules of mucus (gl) from the base of the foot, to aid in locomotion over the substrate. Above the radula sheath (in this view) a single well-formed ciliary lobe (cl) is visible. Ciliary lobes are paired organs covered in cilia that beat to generate a flow of water creating a respiratory current under the shell during early post larval development when the respiratory pores have not yet developed. A close up view of the ciliary lobes are shown in Figure 24 the sensory tubercles (st) projecting from the cephalic tentacles are well resolved in this view. Protrusions growing between the mantle and the foot along the sides of the foot indicate the formation of new epipodial tentacles. When healthy 14-day post larvae were viewed under the light microscope, two epipodial tentacles were visible developing. At 14- days post settlement the operculum had been lost in all of the specimens observed.
Figure 23: *Haliotis australis*; Close up ventral view of a 14-day post settlement larva.

(f) foot; (et) epipodial tentacle; (rs) radula sheath; (ct) cephalic tentacle; (ma) mantle; (gl) globules of mucus.

Figure 24: *Haliotis australis*; View of the ciliary lobes between the cephalic tentacle and the foot.

(f) foot; (rs) radula sheath; (st) sensory tubules; (ct) cephalic tentacle; (ma) mantle; (cl) ciliary lobe.
3.2.7.5 Observations 17-Days Post Settlement

Figure 25 shows three views of the developmental form of the peristomal shell in 17-day post settlement post larvae. The ventral view (lower right Figure 25) shows what appears to be a severely starved post larva (R. Roberts pers. com. 2000) and there appears to be significant difference between the size of the soft body of the abalone and the peristomal shell, however there is a possibility that shrinkage of the soft body is partly due to processing of the samples for examination under the SEM.

Observations under the light microscope of healthy post larvae at this stage showed three pairs of epipodial tentacles developed that were well differentiated. The mean shell length of post larva at 17-days post settlement was 586 μm with an average daily increase of 29.3 μm at 15.4°C since 14-days post settlement (Figure 8).

Figure 25: *Haliotis australis*; Dorsal, ventral and lateral views of 17-day post settlement post larvae.

(f) foot; (ls) larval shell; (ps) peristomal shell; (ct) cephalic tentacle; (rs) radula sheath.
Figure 26 shows an unusual view of the visceral mass of the developing (17-day post settlement) post larva without a shell. Eleven sensory tubercles have formed on the cephalic tentacles and these are shown are fully extended. A ciliary lobe (cl) can be seen at the base of the post larva’s right cephalic tentacle. One epipodial tentacle (et) is present in this specimen. The foot (f) has a broad mass of cilia covering its surface. At the anterior margin of the foot a band of longer cilia are present. The mantle (m) that secretes new shell is drawn away to the top right in Figure 26.

Figure 26: Haliotis australis; 17-day post settlement post larva separated from its shell.

(f) foot; (rs) radula sheath; (et) epipodial tentacle; (ct) cephalic tentacle; (ma) mantle; (cl) ciliary lobe; (d) diatoms.
3.2.7.6 Observations 21-Days Post Settlement

Mass mortalities of post larvae were observed in all settlement tanks from 17-21 days post settlement which suggest that this period of peristomal shell growth is critical in the post settlement survival of *Haliotis australis*. Many larvae were observed to have difficulty moving amongst the diatom biofilms that had developed in the tanks. Rapid growth of diatom biofilms within settlement tanks may be an important factor in the cause of the mass mortalities of larvae during this period, and it may be that post larvae may have been starving even though they were surrounded by well-established diatom biofilms. At 21-days post settlement the mean shell length of post larva was 631 μm, with an average daily increase of 1.3 μm since the last measurement at an ambient seawater temperature of 17.8°C (Figure 8). Figure 27 shows a decaying 21-day post settlement post larva. The soft body of the larva is decaying, and has withered. An important feature of shell development to note is the growth of the peristomal shell over the larval shell. The growing edge of the peristomal shell has become broad and flat as it grows over the larval shell. Overlapping of the larval shell may be an energy expensive process that may stretch the resources of the developing larvae, as a lot of new nacre must be laid over the exposed larval shell, and may be a contributing factor to the mass mortalities observed during this period.

![Figure 27: *Haliotis australis*; Ventral view of a dead and decaying 21-day post settlement post larva.](image)

(f) foot; (ls) larval shell; (ps) peristomal shell.
Figure 28 shows a close up of a small ciliated protozoan often observed in culture tanks. Ciliates are found almost anywhere there is water and it is likely that they were introduced into culture tanks through the hatchery water supply. Most ciliates are heterotrophs, feeding on smaller organisms, such as bacteria and algae, and detritus swept into the oral groove (mouth) by modified oral cilia (Lynn, 2008). The ciliates that were observed in the experiments were between 10 - 20 µm in length and were highly mobile, swimming in a rapid spiral motion. Ciliates were often associated with dead or dying post larvae and ciliates swarming around and under the shell of healthy post larvae appeared to cause the post larvae distress.

Figure 28: Close up view of a ciliate adjacent to the foot of a decaying post larva.

(c) ciliate.
3.2.7.7 Observations 28-Days Post Settlement

In the view of the 28-day post settlement post larva shown in Figure 29, the peristomal shell growth can be observed to have almost completely overlapped the larval shell. The foot has developed, becoming much broader and flatter. Three epipodial tentacles have developed on each side of the foot, and a fourth epipodial tentacle is developing. The cephalic tentacles are contracted (ct) and are bristling with sensory tubercles. The ‘eyes’ (e) are visible as a growth next to the cephalic tentacles. The mantle (m) is retracted and it surrounds the body of the post larva and is most visible forward of the snout (s). The mean shell length at 28-days post settlement was 1.11 mm with an average daily increase of 68.2 μm at ambient seawater temperatures of 18.4°C (Figure 8). Between the snout and the foot, small tufts or bundles of cilia (cilia bundles (cb)) can be seen close to the two ciliary lobes.

Figure 29: Haliotis australis; Ventral view of a healthy 28-day post settlement post larva.

(f) foot; (et) epipodial tentacle; (cl) ciliary lobe; (ct) cephalic tentacle; (cb) cilia bundles; (rs) radula sheath; (ma) mantle.
A close up view of the area between the snout and the foot in Figure 30 shows the cilia bundles in higher resolution. Cilia bundles between the foot and the snout are thought be associated with the ciliary lobes in maintaining respiratory currents. The ciliary bundles may function as current generators to maintain a current of water from under the shell to the ciliary lobes. The cilia bundles may also have a function to help maintain the area between the snout and foot free of debris.

Figure 30: *Haliotis australis*: Cilia bundles between the foot and the snout of juvenile.

(f) foot; (cb) cilia bundles; (rs) radula sheath.

3.2.7.8 Observations 33-Days Post Settlement

Figure 31 shows a dorsal view of the shell of a 33-day post settlement post larva in which the growth of peristomial shell has completed one complete spiral cycle and the larval shell has been completely under-lapped. Spiral shell growth is evident and periods of shell secretion can be seen as growth ‘rings’ close to the larval shell in Figure 31 and these ‘rings’ are more resolved in the view of the larval shell surrounded by peristomial shell growth in Figure 32. Bursts of shell growth, can be seen on the peristomial shell by following the growth lines back from the larval shell in Figure 32. Observation of the larval shells under the light microscope showed two distinct bands of pigmentation on the shell (not visible in SEM) forming on the leading edges of the growing shell. The mean shell length of 33-day post settlement post larvae was 1.34 mm with an average daily increase in shell length of 45.5 μm at
ambient seawater temperatures of 19.3°C since last measurement (Figure 8). The post larvae were able be clearly seen in the experimental tanks with the naked eye at 33-days post settlement.

Figure 31: Haliotis australis; dorsal view of the shell of 33-day post settlement post larva.

(ls) larval shell; (ps) peristomal shell.

Figure 32: Haliotis australis; dorsal view of the shell of 60-day post settlement juvenile shell showing shell texture and growth rings.

(ls) larval shell.
The characteristic anatomical features of the anterior of a 33-day post settlement *Haliotis australis* post larva are well resolved can be seen in the ventral view shown in Figure 33. The eye is clearly visible and is located near the base of the cephalic tentacle. Two ciliary lobes are located adjacent to the cephalic tentacle and the ciliary bundles are clearly visible. The snout (oral disc) is the opening to the radula sac which contains the radula. The radula has rows of teeth that are well developed for scraping the tank surfaces for the biofilm (diatom / mucus / bacteria) food source.

Development of the cephalic tentacles (Figure 34) and epipodial tentacles (Figure 35) is advanced, with many sensory tubercles (st) present. Figure 36 clearly shows that a cluster of sensory hairs (sh) top each of the sensory tubercles. Figure 37 shows a ventral view of a 33-day post settlement post larva. The key features of development in this view are four of epipodial tentacles that are well formed and visible on both sides of the foot, with two new epipodial tentacle ‘buds’ forming. The epipodial tentacles are well developed and covered in sensory tubercles. The epipodial ‘skirt’ between the epipodial tentacles is beginning to flatten and take on broader growth form. The foot is broad and flat (curled up in this view) and covered with cilia. A close up of the anterior region of this view can be seen in Figure 33. The mantle that surrounds the body is the mass of tissue responsible for secreting the peristomal shell. Peristomal shell growth shows strong ridging on the flattened underside of the shell.

*Figure 33: Haliotis australis; Close up view of the head of a 33-day post settlement post larva.*

(f) foot; (cl) ciliary lobe; (cb) cilia bundles; (ct) cephalic tentacle; (rs) radula sheath; (ma) mantle; (et) epipodial tentacle
Figure 34: *Haliotis australis*: View of a cephalic tentacle of a 33-day post settlement post larva covered in sensory tubercles.

(st) sensory tubule; (rs) radula sheath.

Figure 35: *Haliotis australis*: Close up view of the edge of the foot and showing epipodial tentacles covered with sensory tubercles.

(f) foot; (et) epipodial tentacles; (st) sensory tubercles.
Figure 36: *Haliotis australis*; Close up view of the sensory tubercles that are located on the cephalic and epipodial tentacles. Each tubercle is topped with a bundle of sensory hairs.

(st) sensory tubercles; (sh) sensory hairs.

Figure 37: *Haliotis australis*; Ventral view of 33-day post settlement post larva.

(f) foot; (et) epipodial tentacle; (cl) ciliary lobe; (ct) cephalic tentacle; (rs) radula sheath; (ma) mantle.
3.2.7.9 Observations 39-Days Post Settlement

Figure 38 shows a ventral view of the developing post larvae at 39-days post settlement. The shell development has reached the ‘notch stage’ which indicates the formation of the first respiratory pore (rp). The mantle (m) shows differentiation near the respiratory pore, forming two flaps or the mantle notch (mn) that enable the mantle to form the respiratory pores in the shell and allow the passage of the respiratory current through the mantle and out of the respiratory pore. The ciliary lobes (cl) are still present, as are the ciliary bundles (cb) between the snout and the foot. The sensory cephalic tentacles are retracted in this view. New shell secretion can be seen as smooth shell over the sharp ridging at the posterior of the animal.

![Figure 38: Haliotis australis; Ventral view of 39-day post settlement post larva with 1st respiratory pore forming.](image)

(f) foot; (et) epipodial tentacle; (mn) mantle notch; (rp) respiratory pore; (ma) mantle; (rs) radula sheath; (ct) cephalic tentacle.

3.2.7.10 Observations 41-Days Post Settlement

Formation of the first respiratory pore was observed to be complete at 41-days post settlement and development of the respiratory pore marks the end of the ‘post larval’ stage of development and the post larva can be described as a ‘juvenile’. Mean shell length at 41-days post settlement was 1.65 mm, with an average daily increase in shell length of 39.0 µm at ambient temperatures of 19°C since 33-days development (Figure 8). Five to seven pairs of epipodial tentacles have developed on each side of the animal, and the snout shows more complex development.
3.2.7.11 Observations 45-Days Post Settlement

Figure 39 shows a view of a 45-day post settlement *H. australis* juvenile. The broad foot is contracted revealing ten epipodial tentacles on each side of the foot, with more epipodial buds developing. Between the snout and the foot the ciliary lobes are still visible. Close to the respiratory pore the mantle has developed a split allowing it to shape the respiratory pores.

![Figure 39: *Haliotis australis*; Ventral view of 45-day post settlement juvenile abalone.](image)

(f) foot; (et) epipodial tentacle; (rp) respiratory pore; (cl) ciliary lobe; (rs) radula sheath; (ma) mantle; (ct) cephalic tentacle.

At 45-days post settlement two respiratory pores (rp) have been formed (the second respiratory pore is visible in Figure 39). At this stage healthy *H. australis* juvenile are observed to be highly mobile and have three bands of olive and green pigmentation present on the dorsal shell surface when observed under the light microscope. The mean shell length of the juveniles at 45-days post settlement was 1.99 mm with an average daily increase in shell length of 85.1 µm at ambient seawater temperatures of 19.5°C since last measurement (Figure 8).
3.2.7.12 Observations 52-Days Post Settlement

The notch of a new respiratory pore can be seen forming in a view of a 52-days post settlement juvenile in Figure 40, and the mantle shows a split along the axis of the respiratory pore called the mantle notch (mn) and is clearly visible. At 52-days post settlement three respiratory pores were observed to have fully formed and in some individuals a fourth respiratory pore was developing. A ciliary lobe (cl) can still be seen in this 52-day post settlement juvenile. The foot is broad in relation to the snout (oral disk). Epipodial development is advanced with at least ten tentacles developed on each side and up to seven more developing.

Figure 40: *Haliotis australis*; Ventral view of 52-day post settlement juvenile.

(f) foot; (et) epipodial tentacle; (ma) mantle; (rp) respiratory pore; (mn) mantle notch; (rs) radula sheath; (ct) cephalic tentacle.

The epipodium or the broad fold of tissue that will become the epipodial skirt in the adult abalone is well differentiated and observed as the band of tissue extending from the foot to the base of the epipodial tentacles (et). At 52-days post settlement the mean shell length observed was 2.65 mm (Table 2) with an average daily increase in shell length of 94.9 μm at ambient seawater temperatures of 20.5°C since last measurement at 45-days post-settlement.
3.2.7.14 Observations 60-Days Post Settlement

Figure 41 shows the dorsal view of the development of a juvenile *H. australis* at 60-days post settlement. Five respiratory pores have formed and the juvenile shell now has the typical ‘ear’ shaped shell found in juvenile and adult abalone. The spire of the shell is located posteriorly and is centered on the larval shell (ls). Respiratory pores form and are distributed along the left hand border of the shell. The mineralized parts of the shell are initially laid down by the mantle, as calcium carbonate crystals within the confines of organic (conchiolin) envelopes. Further development of the shell is made up of an outer (prismatic) layer of calcite crystals and an inner (nacreous) layer of aragonite crystals. The ‘nacre’ is what gives the inner surface its iridescent appearance. An external protein sheath called the periostracum covers the outer layer of the shell and contains the pigments that give the outer shell its colour. The larvae appeared pink in colour when viewed with the naked eye. Examination of juveniles under the light microscope show that colour bands appear intermittently in the same position of the growth rings. Mean shell length at 60-days post settlement was 3.3 mm with an average daily increase of 80.9 μm at ambient seawater temperatures of 21°C since the last measurement taken at 52-days post settlement (Table 2).

![Figure 41: Haliotis australis; dorsal view of the shell of a 60-day post settlement juvenile.](image)

(ls) larval shell; (rp) respiratory pore.
Figure 42: *Haliotis australis*; Oblique Dorsal view of a 60-day post settlement juvenile showing the elevated respiratory pores, shell ridges and larval shell.

(ls) larval shell; (rp) respiratory pore; (sr) shell ridges.

The oblique view of a 60-day post settlement *H. australis* juvenile in Figure 42 shows the respiratory pores as the prominent features of the external shell. The first respiratory pore is formed as the mantle separates at the anterior margin of the shell, forming the mantle notch. This creates a slit, giving rise to an opening or pore on the growing surface of the shell. Additional pores are formed as the shell increases in size. After four or five respiratory pores are formed the first respiratory pore is closed. As the abalone grows only four or five respiratory pores are open at any time. The elevated tremata of the respiratory pores may passively serve to enhance respiratory exchange in areas that experience low water movement (i.e. culture tanks). The shell ridges (sr) near the base of the shell may serve a function of providing an anchoring point during feeding. The view across the surface of the shell in Figure 42 provides a perspective of the undulating contour of the peristomal shell as it radiates out from the central spire at the larval shell (ls). The image on the title page of this thesis shows a close up of a respiratory pore at 60-days post settlement.
The main features of the 60-day post settlement juvenile *H. australis* are able to be clearly seen in the ventral view of the juvenile in Figure 43. The massive muscular foot (f) occupies most of the internal concave surface of the shell. The epipodium is visible as a sheet of tissue that extends from the dorsal part of the foot; it supports epipodial tentacles (et) that emerge from notches on the fringed surface. The mantle (m) is a flap of tissue, which lies between the body and the shell. The mantle is responsible for secreting new shell growth and formation of the respiratory pores. The cephalic region supports the snout, a pair of cephalic tentacles (ct), and the eyestalks (not visible in this view). The snout contains the mouth is a dorso-ventral slit, surrounded by ridges, which form the snout (s) or oral disc. A close up of the snout is shown in Figure 44 and a view of the radula is visible in Figure 45.

**Figure 43: Haliotis australis; Ventral view of the foot, mantle and shell of a of a 60-day post settlement juvenile.**

(f) foot; (et) epipodial tentacle; (ma) mantle; (mn) mantle notch; (rp) respiratory pore; (ct) cephalic tentacle; (rs) radula sheath; (r) radula.
Figure 44: *Haliotis australis*; the ridged radula sheath (snout or oral disc) of a 60-day post settlement juvenile, with the central mouth containing the radula.

(et) epipodial tentacle; (cb) cilia bundle; (ct) cephalic tentacle; (ma) mantle; (rs) radula sheath.

Figure 45: *Haliotis australis*; Close up view of the mouth of a 60-day post settlement juvenile showing the oral disc with the radula partially extended.

(mt) marginal teeth; (lt) lateral teeth; (rt) rachidian teeth.
The radula is a highly developed feeding organ consisting of a ribbon of chitonous teeth attached to a cartilaginous base called the odontophore. The radula acts as a rasp to scrape the surface of the substrate and also as a conveyor of food (usually benthic diatoms in post larvae and biofilms and seaweeds in juveniles and adults) through the mouth opening and into the oesophagus. The radula teeth are arranged in rows. On each row of the radula teeth there is a single central rachidian tooth which is flanked by up to five rows of lateral teeth and several rows of outer marginal teeth. The rachidian tooth, lateral teeth and marginal teeth differ from one another in shape and function. The different radula teeth of *H. australis* are shown in Figure 46.

![Figure 46: *Haliotis australis*; Close up view of the radula of a 60-day post settlement juvenile.](image)

(mt) marginal teeth; (lt) lateral teeth; (rt) rachidian teeth.

In the background of Figure 46 a large rachidian tooth (rt) can be seen with the large solid lateral teeth (lt) visible in the mid-ground and the fine marginal teeth (mt) in the foreground. The rachidian tooth and lateral teeth of the radula are used for grinding and crushing the frustules of attached diatoms and other encrusting organisms before the rake-like marginal teeth sweep the crushed diatoms and biofilm into the mouth as the odontophore muscle retracts the radula into the radula sheath.
3.3 Results Part 2 - Factors Affecting Settlement

3.3.1 Overview

The following section reports the results of the findings of the experiment conducted to investigate factors affecting the settlement of *Haliotis australis*. The aim of the settlement experiment was to assess the effectiveness of use of established diatom biofilms, as used by some hatcheries for other abalone species, to induce settlement of *H. australis* larvae.

The following results section presents a description of the ungrazed 8 day old diatom biofilm used in the experiments and the results of the SEM analysis undertaken on the biofilm, followed by general observations of the behaviour of the *H. australis* larvae within each of the four diatom biofilm treatments during the ‘settlement period’ of the experiment. The settlement period was between (day-0 to day-4) when competent ‘swimming’ and ‘crawling’ larvae were retained within the experimental tanks by the use of banjo sieves placed over the tank outlets. The experimental treatments to be tested were established biofilms (‘8 Day Ungrazed Biofilm’), established diatom biofilms pre-grazed by conspecific adults to produce mucus trails (‘8 Day Grazed Biofilm’), newly established diatom biofilms (‘1 Day Biofilm’), and no diatom biofilm treatments (‘No Biofilm’) that were used as a control. Half of the experimental treatment replicates were also subjected to a treatment of gamma-aminobutyric acid (GABA) immediately prior to introduction to the settlement tanks to test the effectiveness of GABA as a chemical settlement inducer.

3.3.2 Diatom Biofilm – Scanning Electron Microscopy Analysis

Mixed diatom biofilms were commonly used as the primary settlement substrate for the settlement of the Blackfoot Paua *Haliotis iris* at the Rainbow Abalone Ltd. hatchery. The purpose of the experiment was to test suitability of the diatom biofilms as a settlement substrate for *H. australis* larvae. The experimental ‘8 Day Ungrazed Biofilm’ and ‘8 Day Grazed Biofilm’ replicate tanks were seeded with a diatom slurry eight days using techniques typically employed within the hatchery prior to the introduction of competent veliger larvae of *H. australis* into the experimental tanks. A sample of the biofilm was collected on a glass slide placed within the experimental tanks and was later analysed using SEM. At 8 days old the biofilm was clearly visible as a light green film on tank surfaces. Upon examination with of the biofilm with a light microscope the majority of the diatoms observed were small pennate diatoms between 5-10µm in length. SEM analysis shows the biofilm to be a complex matrix of bacteria and diatoms. Figure 46 and Figure 47 show that the biofilm at the start of the experiment was approximately 1 - 2 cells thick, having a mixed species composition of mainly pennate diatoms and bacteria embedded in a matrix polysaccharide mucus (a product of diatom photosynthesis) exuded from the diatoms.
Figure 47: Oblique view of an 8 day old diatom biofilm from a glass slide placed within the experimental settlement tank.

(d) pennate diatoms.

Figure 48: Close up of an ‘8-day Ungrazed Biofilm’ settlement substrate taken from within experimental tanks used for the settlement of *Haliotis australis* larvae.

(pm) polysaccharide mucus; (b) bacteria; (d) pennate diatoms.
3.3.3 Observations from ‘8 Day Grazed Biofilm’ Replicates

Each of the settlement tank replicates in this diatom film treatment were established with an 8 day diatom biofilm before being pre-grazed by two adult *H. australis* for 48 hours prior to settlement. Approximately 40-50% of the diatom film within the settlement tank surfaces were grazed by the adult abalone. The diatom biofilm was observed to have been disturbed, the presence of a characteristic zig-zag pattern caused by the radula action of the adult abalone. The radula feeding patterns could be easily seen amongst the olive green colored biofilm and occurred on most of the tank surfaces, suggesting that the adult abalone had moved over all of the settlement tank surface and left mucus trails throughout. Trails of pedal mucus were also visible in tanks, and a sample of the pedal mucus was extracted from the tank, stored and later observed using the SEM (Figure 51).

![Mucus trail](image)

**Figure 49:** Pedal mucus trail from the adult *H. australis* abalone used to pre-graze the experimental replicates and pennate diatoms from the diatom biofilm.

(mt) mucus trail; (d) diatoms.

The GABA treated larvae appeared to settle within 30 minutes of being added to the ‘8 Day Grazed Biofilm’ settlement tanks. Observations of untreated ‘8 Day Grazed Biofilm’ replicates showed a strong settling response, with larvae visibly testing the substrate on areas cleared of the diatom biofilm by the pre-grazing by the conspecific adults.

Observations and samples taken from the settlement tanks in both GABA treated and untreated ‘8 Day Grazed Biofilm’ replicates showed that the velum was still present in crawling larvae, indicating an
ability to return to the water column. When air flow was restored to the settlement tanks at day 3 of the experiment, many of these larvae that appeared to have settled returned to the water column and began swimming. Larvae in ‘8 Day Grazed Biofilm’ replicates that had settled and metamorphosed tended to attached to the tanks surfaces near the air/water interface.

3.3.4 Observations from ‘8 Day Ungrazed Biofilm’ Replicates.

Observation of the behaviour of competent *H. australis* larvae, when introduced into the tanks with a diatom biofilm that had been grown for 8 days, showed a rapid attachment and settlement response. Larvae quickly settled and began crawling and searching behaviour on the substrate. In the replicates that had been treated with GABA, no larvae were observed to be swimming in the water column after 30 minutes. In the untreated ‘8 Day Ungrazed Biofilm’ replicates the settling response was slower that that seen in GABA treated replicates however all of the veliger larvae appeared to have settled when observed after 24 hours.

Samples of the settled larvae taken from both the GABA treated and untreated ‘8 Day Ungrazed Biofilm’ replicate tanks up until the first drain and fill on day 4 of the experiment showed many of the larvae still retained the velum, indicating that metamorphosis had not yet occurred. The larvae retained an ability to return to the water column particularly when the tanks were disturbed by handling, or by the turbulence caused by aeration from the air-stone or inlet water. Switching on the lighting above the settlement tanks also triggered a swimming response from larvae that had previously appeared to be attached to the substrate and crawling. Observations under the light microscope of larvae taken from different locations within the settlement tanks identified that settled larvae that had undergone metamorphosis (as indicated by loss of the velum and with peristomal shell growth) were most often concentrated near the water/air interface of the experimental settlement tanks.

3.3.5 Observations and Results from ‘1 Day Biofilm’ Replicates

‘1 Day Biofilm’ replicates were prepared by adding a diatom slurry to the settlement tanks 24 hours prior to the introduction of the competent veliger larvae into the settlement tanks.

The surfaces of the ‘1 Day Biofilm’ settlement tanks appeared clean with no visible diatom biofilm present when larvae were introduced into the tanks. After four days post settlement a faint green colour was observed on the tank surfaces indicating that a diatom biofilm was developing.

The veliger larvae in GABA treated ‘1 Day Biofilm’ replicates appeared to have initiated an attachment and settlement response within the first 30 minutes of being introduced into the settlement tanks, with no larvae being observed swimming. Observations of the tanks after 24 hours identified that many of
the GABA treated veliger larvae had returned to the water column and a large number of larvae were observed swimming until day 4 when the tanks were drained and filled.

Observations of larval behaviour in untreated ‘1 Day Biofilm’ replicates, showed no distinct initial settlement response and very few of the larvae exhibited attachment or searching behaviour that may have indicated settlement. Observations of the settlement tanks three days after introduction of the larvae into the tanks air flow was re-established, identified many veliger larvae swimming near the 80 µm mesh screen banjo sieves connected to the tank outflow pipes.

### 3.3.6 Observations from ‘No Biofilm’ Replicates

In untreated ‘No Biofilm’ replicates, most of the veliger larvae were observed to remain swimming in the water column until day 4 when they were removed from the system by routine drain and fill maintenance of the settlement tanks. There appeared to be no settlement response in untreated no biofilm replicates during the first four days after introduction of larvae to tanks. The larvae remained actively swimming.

Observations of GABA treated ‘No Biofilm’ replicates showed that the veliger larvae appeared to settle within 30 minutes of treatment. However after three hours most of the larvae had returned to the water column and had resumed swimming. ‘No Biofilm’ experimental settlement tanks were naturally seeded with diatoms from seawater entering the experimental system through the main hatchery water supply. After seven days a green film was observed to be developing on the white tank surfaces.

Ciliate blooms were observed adjacent to the banjo sieve at the water outflows in all replicates with large numbers being present swarming over the filter mesh and also amongst the settled post larvae.
3.3.7 General Experimental Results - Tank Counts at 33 Days

Full tank counts in all experimental replicates were conducted at 33-days post settlement to analyse and evaluate the success of each induction treatment. Additional full tank counts were undertaken at 39 days, 45 days, 52 days and 60 days post settlement to measure survival through time.

Figure 49 presents the data showing survival of *Haliotis australis* post larvae within all 40 experimental replicate settlement tanks at 33-days post settlement.

![Graph showing survival of Haliotis australis at 33 days post settlement for all treatments.](image)

**Figure 50: Total counts of survival of Haliotis australis at 33 days post settlement for all treatments.**

*(Each bar represents one of the settlement tank replicates in each experimental factor).*

A total of 3600 individual post larvae survived at 33-days post settlement. In most of the experimental tanks counts were generally low (< 50 individual *Haliotis australis* surviving) with only 14 of the 40 replicates in the experiment having 50 or more post larvae surviving at the end of the 60 day experiment. The highest counts of *H. australis* post larvae at 33-days post settlement was observed in a GABA treated ‘8 Day Grazed Biofilm’ replicate (495 post larva). The replicate with the lowest number of *H. australis* post larvae surviving at 33-days post settlement was an untreated ‘1 Day Biofilm’ replicate (6 post larva).
3.3.8 Statistical Analysis of Survival at 33 Days Post Settlement Data

Differences in population / count data of *H. australis* at 33-days post settlement between the diatom film treatments (‘8 Day Biofilm’, ‘Grazed 8 Day Biofilm’, ‘1 Day Biofilm’ and ‘No Biofilm’ and the GABA / no-GABA treatments, were examined with analysis of variance (ANOVA), using Minitab 16 (Minitab Inc., Pennsylvania, USA).

The Anderson-Darling test for normality and Bartlett’s test for equal variance were conducted prior to ANOVA analysis to ensure that the data complied with the assumptions of normality and homogeneity of variance and the results of these two tests are presented in Table 2.

Results of the Anderson-Darling test identified that the raw data was not normally distributed. and the data was subsequently Log<sub>10</sub> transformed and satisfied the assumptions of normality and homogeneity of variance. ANOVA analysis was conducted on the Log<sub>10</sub> transformed data (Table 3).

When significant differences were detected (p-value < 0.05), multiple comparisons between the relevant means were conducted using Tukey’s method (equal number of observations per treatment) (Table’s 4 and 5).

Anova using Log<sub>10</sub> at 33-days post settlement data found a significant difference between the different diatom film treatments (F<sub>3, 32</sub> = 6.64, P-value < 0.001). Pairwise comparisons (Table 4) showed that the mean count total of the ‘8 Day Grazed Biofilm’ treatment was significantly higher than the means of ‘1 Day Biofilm’ and ‘No Biofilm’ treatments. The ‘8 Day Ungrazed Biofilm’ mean was also significantly higher than ‘No Biofilm” treatment’.

No significant difference was found between the GABA treatment and no-GABA treatment (F<sub>2, 32</sub> = 4.04, P-value = 0.053 ), however it is interesting to note that the P-value is very close to 0.05 suggesting that the threshold at which a significant difference may detected between the treatments was close. This result is likely to have been driven by the results from one high count within the ‘8 Day Ungrazed Biofilm’ replicates. Figure 51 shows what appears to be a big difference between GABA and no-GABA in ‘8 Day Ungrazed Biofilm’ treatments.

No significant interaction was found between the two factors (GABA treatment and Diatom Biofilm treatments) (F<sub>3, 32</sub> = 2.44, P-value = 0.082).
TABLE 2: RESULTS OF ANDERSON-DARLING TEST FOR NORMALITY AND BARTLETT'S TEST FOR EQUAL VARIANCE.

<table>
<thead>
<tr>
<th></th>
<th>Bartlett's Test</th>
<th>P - value</th>
<th>Anderson – Darling</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>33-day post settlement</td>
<td>61.43</td>
<td>&lt;0.005</td>
<td>3.068</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Log_{10} 33-day post settlement</td>
<td>17.66</td>
<td>0.014</td>
<td>0.566</td>
<td>0.133</td>
</tr>
</tbody>
</table>

TABLE 3: TWO FACTOR ANOVA ANALYSIS OF LOG_{10} TRANSFORMED DAY-33 SURVIVAL DATA.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Type</th>
<th>Levels</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA Treatment</td>
<td>fixed</td>
<td>2</td>
<td>GABA, No-GABA</td>
</tr>
<tr>
<td>Diatom Biofilm Treatment</td>
<td>fixed</td>
<td>4</td>
<td>8 Day Grazed Biofilm, 8 Day Ungrazed Biofilm, 1 Day Biofilm, No Biofilm</td>
</tr>
</tbody>
</table>

Two Factor ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA Treatment</td>
<td>1</td>
<td>0.4216</td>
<td>0.4216</td>
<td>0.4216</td>
<td>4.04</td>
<td>0.053</td>
</tr>
<tr>
<td>Diatom. Film Treatment</td>
<td>3</td>
<td>2.0765</td>
<td>2.0765</td>
<td>0.6922</td>
<td>6.64</td>
<td>0.001</td>
</tr>
<tr>
<td>Treatment*Diatom. Film Treatment</td>
<td>3</td>
<td>0.7639</td>
<td>0.7639</td>
<td>0.2546</td>
<td>2.44</td>
<td>0.082</td>
</tr>
<tr>
<td>Error</td>
<td>32</td>
<td>3.3379</td>
<td>3.3379</td>
<td>0.1043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>6.5999</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 4: GROUPING DIATOM BIOFILM TREATMENT INFORMATION USING TUKEY METHOD AND 95.0% CONFIDENCE.

<table>
<thead>
<tr>
<th>Diatom Biofilm Treatment</th>
<th>N</th>
<th>Mean</th>
<th>Grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 Day Grazed Biofilm</td>
<td>10</td>
<td>2.016</td>
<td>A</td>
</tr>
<tr>
<td>8 Day Ungrazed Biofilm</td>
<td>10</td>
<td>1.916</td>
<td>A B</td>
</tr>
<tr>
<td>1 Day Biofilm</td>
<td>10</td>
<td>1.568</td>
<td>B C</td>
</tr>
<tr>
<td>No Biofilm</td>
<td>10</td>
<td>1.473</td>
<td>C</td>
</tr>
</tbody>
</table>

*Means that do not share a letter are significantly different.

TABLE 5: GROUPING GABA TREATMENT INFORMATION USING TUKEY METHOD AND 95.0% CONFIDENCE.

<table>
<thead>
<tr>
<th>GABA Treatment</th>
<th>N</th>
<th>Mean</th>
<th>Grouping*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>20</td>
<td>1.846</td>
<td>A</td>
</tr>
<tr>
<td>Untreated (No-GABA)</td>
<td>20</td>
<td>1.640</td>
<td>A</td>
</tr>
</tbody>
</table>

*Means that do not share a letter are significantly different.
Figure 51: Mean count ± SE, of *H. australis* post larvae for all experimental treatments at 33-days, 39-days, 45 days, 52-days and 60-days post settlement. (Dark bar = no GABA / light bar = GABA treatment).
3.3.9 Mean Survival from 33 Days to 60 Days Post Settlement.

The mean count of *H. australis* post larvae for each diatom biofilm treatment was calculated for each of the full tank count data sets at 33, 39, 45, 52 and 60 days post settlement. Figure 5 presents a series of bar graphs showing mean counts of *H. australis* post larva for GABA treated and untreated (no-GABA) experimental diatom biofilm treatments. Each of the biofilm treatments is discussed below.

**‘8 Day Grazed Biofilm’** - Biofilms that had been pre-grazed with conspecific adults had high survival (> 150 post larvae surviving) at 33-days post settlement in both untreated (no-GABA) and GABA treated experimental tanks. The mean count of post larvae within the no-GABA ‘8 Day Grazed Biofilm’ tanks at 33-days post settlement was 174 post larvae (SE = 71.21) this is compared with a mean count of post larvae of 166 (SE=85.46) within GABA treated ‘Grazed 8 Day Biofilm’ tanks which was largely driven by a high count of 495 individuals being recorded in one of the GABA treated replicate tanks.

The survival rate of the juvenile *H. australis* in untreated no-GABA ‘8 Day Grazed Biofilm’ tanks through to the end of the experiment was approximately 65% with a mean count of 100 (SE=36.37) juveniles surviving when the final tank counts were undertaken at 60-days post settlement (the survival rate at 60-days ranged from 43% - 86% within these replicate tanks). This compared to a survival rate of approximately 64% within GABA treated ‘8 Day Grazed Biofilm’ tanks where a mean count of 80 juveniles (SE=25.65) survived the period from 33-days post settlement to 60-days post settlement. It is interesting to note that in the most densely settled tank (495 individuals) had the lowest survival rate (31%) of all of the experimental tanks.

**8 Day Ungrazed Biofilm Replicates** – GABA treated ‘8 Day Ungrazed Biofilm’ produced the highest mean counts in all of the experimental treatments with a mean count of 191 post larvae (SE=41.25) surviving at 33-days post settlement. This treatment had a mean survival rate of 67% with 121 juveniles (SE=17.32) surviving the period from 33 days until 60 days post settlement.

Untreated no-GABA‘8 Day Ungrazed Biofilm’ replicates had a low mean count (<50 post larvae surviving) with a mean of 40 post larvae (SE = 6.65) surviving at 33-days post settlement. However a relatively high survival rate of approximately 73% was recorded in the untreated ‘8 Day Ungrazed Biofilm’ treatment with a mean count of 29 juveniles (SE=5.17) surviving until 60-days post settlement.

**‘1 Day Biofilm’ Replicates** - The mean count within the untreated no-GABA ‘1 Day Biofilm’ treatment at 33-days post settlement was low with a mean of 38 post larvae (SE = 9.16) surviving. A mean count of 29 juveniles (SE=6.20) survived until the final tank counts at 60-days post settlement, achieving the highest survival rate of approximately 84%.
The mean count within GABA treated ‘1 Day Biofilm’ replicates was slightly better, but still low, with a mean count of 47 post larvae (SE=4.91) at 33-days and a mean count of 32 juveniles (SE=3.13) surviving at 60-days post settlement, recording a survival rate of approximately 69% for the period.

‘No Biofilm’ Replicates – Low mean counts were also recorded within untreated no-GABA ‘No Biofilm’ replicates with a mean count of 35 post larvae (SE = 6.81) surviving at 33-days post settlement and a mean count of 29 juveniles (SE=8.15) surviving until 60-days post settlement, producing a high survival rate of approximately 83% (from 33 to 60 days).

The mean count within the GABA treated ‘No Biofilm’ replicates was recorded as 30 post larvae (SE=4.49) at 33-days post settlement with a mean count of 17 juveniles (SE=3.12) surviving until 60-days post settlement which resulted in a relatively low survival rate of approximately 59% (from 33 to 60 days) compared with other treatments where densities of post larvae within the tanks were initially recorded as being low.

Survival

Figure 52 shows a scatter plot of the survival rates for all experimental treatments from 33-days to 60-days post settlement. Highest mean survival was achieved in the untreated (no-GABA) ‘1 Day Biofilm’ (84%) and no-GABA ‘No Biofilm’ (83%) treatments. Lowest mean survival was recorded in GABA treated ‘No Biofilm’ (59%) and GABA treated ‘8 Day Grazed Biofilm’ (64%) treatments.

![Figure 52: % Survival of H. australis juveniles for all experimental treatments at 60-days post settlement.](image-url)
Chapter 4 - General Discussion

4.1 Scanning Electron Microscopy Results

SEM has been previously used to elucidate features of larval abalone (Garland & Cooke, 1985; Lu et al., 1987; Auzoux-Bordenave et al., 2010). The SEM analysis of *Haliotis australis* presented in this paper represent development the first major SEM series of photomicrographs examining larval and post larval development for this species. The use of the SEM has enabled a close examination, and documentation, of the fine features of developing *H. australis*, from the gametes, through the stages of the larval development including trochophore, pre-torsion veliger and late veliger larval stages. The use of SEM to examine the growth and development of the post larval stages of *H. australis* has also enabled a close examination of the morphology of post larval and juvenile *H. australis* to 60 days post settlement that are difficult to be clearly resolved using light microscopy. Examination of the scanning electron micrographs of the metamorphosis of *Haliotis australis* highlighted some interesting features of development highlighted below:

- The operculum present in larval *H. australis* is retained in post larval up to 12 days post-settlement, when peristomal growth shows a mid-asymmetric growth form sc ebred by Lieghton (1974).

- High mortality of post larvae occur at the period where the peristomal shell grows under the larval shell for the first time (17-20 days post settlement).

- The mantle diverges at its anterior margin around 33 days post-settlement, and by 39 days the 1st respiratory pore is well developed. Development of the first respiratory pore is complete at 41-days post settlement at ambient temperatures of 15-19ºC.

- After the major mortality periods, and formation of the first respiratory pore, the growth of the foot rapidly increases in relation to the snout, and the number of epipodial tentacles increase.

- Ciliary lobes (important for generating and maintaining ventilation currents in post-larvae) thought to disappear after the formation of the first respiratory pore (Hahn 1989), are still present in larvae of *Haliotis australis* at 60 days post settlement, when five respiratory pores have been formed.

- Strong ridging present on the posterior ventral surface of the peristomal shell is present from the initiation of shell growth. This ridging may be used as a ‘skid’, as the abalone moves
forward on the feed substrate, or as a ‘brake’, holding the abalone in position whilst rasping the feed substrate with its radula. Ridging of the rear margin may also act as a reinforcing surface when laying down new shell growth.

4.1.1 Experimental Factors Affecting Settlement Results

Analysis of the results of post larval survival at 33-days post settlement was used to infer the settlement success at the start of the experiment during the ‘settlement period’ when swimming larvae had an opportunity to attach, settle and begin metamorphosis between (0 to 4-days post settlement). It was difficult to arrive at firm conclusions on determining the best methods, or diatom biofilm settlement substrates for inducing settlement in *Haliotis australis* through inferring settlement success based on survival data measured at 33 days post settlement. Any conclusive argument based only on the raw survival data from these experiments is marginal at best. However a combination of the observations from the experimental (survival) results and direct observations of the experimental tanks enable some reasonable conclusions to be drawn. The high number of mortalities of developing post larva observed during tank maintenance activities at between two to three weeks post settlement in ‘8 Day Ungrazed Biofilm’ and ‘8 Day Grazed Biofilm’ replicates indicate that settlement and metamorphosis in these treatments was initially high. The analysis of the results of survival from 33-days post settlement to 60 days post settlement generally indicated that tanks with higher densities of settled post larvae had the lower survival rates when compared with tanks with initial lower densities.

Both GABA treated and untreated ‘8 Day Grazed Biofilm’ treatments showed high mean counts of surviving post larva ( >150 post larva surviving) at 33-days post settlement with similar results recorded in GABA treated ‘8 Day Ungrazed Biofilm’ treatments, an ANOVA showed that there were significant differences between these treatments and ‘No Biofilm’ treatments. This indicates that the use of a GABA dip immediately prior to settling larvae onto a prepared diatom substrate may be an option to be considered for use in hatcheries attempting to produce commercial numbers of *H. australis* juveniles for grow out. All other treatments had low (<50 post larva surviving) at 33-days post settlement irrespective of the biofilm treatment. Even though the differences shown by the data between GABA treated and untreated larva was not statistically significant (P = 0.053) the results and observations suggest that a GABA treatment has a role in enhancing initial settlement of *H. australis* larva.

4.1.2 Potential Sources of Error and Variation within the Experiment

Due to the size and scale of the experimental setup and the challenges associated with gathering data, a wide range of sources of error is likely to have been introduced to the experiment. Some of the sources of error that is likely to have occurred are:
• Variation in the number of competent larvae that were initially added to each replicate tank at the beginning of the experiment. The total number of larvae were divided between the 40 replicate tanks by splitting a known volume of seawater that containing all of the larvae from the rearing tanks. However it is likely that each of the 40 replicates contained approximately equal quantities of larvae at the beginning of the experiment. At the time there was no method available to quantify the number of larvae in each treatment/replicate.

• There is likely to have been variations in rate of water flow and air supply to experimental replicate tanks arising from the design of the experimental system. The five tiers of experimental tanks were observed at times to have subtle variations in water and air flow rates to individual tanks as a result of pressure drops occurring long the length of the supply lines. The survival of post larvae may have been affected by the experimental tank position within the tier or between tier levels of the experimental system due to subtle variations in lighting, and air and water flows within the tanks.

• Variations in water temperature within replicate tanks during the experiment may have arisen as a result of the position of the tanks within the experimental system. Other than daily seawater temperature measured from the main hatchery system, temperatures within each replicate tank was not undertaken in this experiment. The low water volume within each of the tanks (<10 litres) and lack of temperature control within the experimental room means that water temperatures within the experimental tanks may have been influenced by external sources, such as heat from lighting positioned above the tanks or from ambient daily temperatures within the room housing the experiment.

• Differences between replicates in the composition of diatom species and bacteria present in the biofilm within and between replicate tanks may have had an effect on survival of post larvae including potential starvation of post larvae caused by overgrazing. There was no method available to control diatom composition to ensure that only preferred diatom species were present in the tanks. It is likely that diatom composition changed through time as a result of grazing pressure and possibly preferential species selection by post larvae and by natural seeding of tanks by a range of diatom species present in the water supplied to the hatchery.

4.1.3 Maintaining Diatom Biofilms during Early Post larval Development

The results indicate that close attention to diatom biofilm levels present is required to maintain the supply of adequate diatom feedstock to post larvae of *H. australis* to ensure survival of high initial settlements of larvae. Diatom biofilms were lost from the settlement tanks in a number of ways: by direct consumption of attached diatoms; by dislodgement of the diatom biofilm from substrate by the
action of grazing post larvae/juveniles; and by the excretion of unattached diatoms in feces (fecal pellets were observed to contain intact viable diatoms); and debris within the tank including dislodged diatom biofilm which tended to clump together and remain unattached. The detached diatoms were not available for grazing and were removed from the system at regular drain and fill maintenance. Diatom biofilms were also lost as a result of sloughing off the side of the settlement tanks both by its own accord or as a result of the gentle stream of water used to rinse tanks of mortalities and debris. If the rinse was too hard diatom biofilm and associated juveniles were lost from the tanks, but if the rinse was too gentle mortalities and associated ciliates remained in the tank to contaminate system.

To reduce the loss of diatom biofilms new settlement tanks should be prepared prior to inoculation by weathering the internal surfaces of the tanks using a fine to medium coarse sand-paper prior to final cleaning, sterilisation and diatom inoculation to assist the adhesion of the diatom biofilm. Additional diatoms were seeded into the tanks at between 19-21 days post settlement to arrest a trend of high mortalities occurring during development likely to be associated with density driven starvation. Additional diatoms slurry added to the densely settled tanks showing the most grazing pressure may have yielded higher rates of survival in those tanks at 33-days post settlement.

4.2 Broodstock Conditioning

The Haliotis australis broodstock for this thesis research were bought into condition for spawning at the Rainbow Abalone Ltd hatchery facilities by keeping in darkness and ad libitum feeding of an artificial abalone diet pellets and fresh seaweed (Lessonia variegata) for three months at ambient seawater temperatures (10-15°C). Temperature is important in controlling the reproductive cycle of Haliotis australis and adults can be bought into spawning condition within 21 weeks at 15°C and can be decreased to 18 weeks if seawater temperatures are raised to 18°C for two weeks prior to spawning (Moss, 1998). The readiness of abalone to spawn may be calculated using a method that measures effective accumulated temperature (EAT) in °C hours or days (Seki & Kan-no, 1981b; Uki & Kikuchi, 1984). EAT is calculated by taking the water temperature of the broodstock rearing system (°C) minus the biological zero point (BZP) multiplied by the time (days or hours) of the conditioning period.

Broodstock conditioning experiments undertaken on New Zealand abalone species by Kabir (2001) determined that the biological zero point (BZP) temperature for H. australis is 5.02°C (c.f. 6.21°C for H. iris) and the EAT required to produce a fully ripe and spawnable gonad in H. australis was calculated to be ≥ 1400°C-days (c.f. H. iris ≥ 2700°C-days). The same experiments found the most rapid gonad growth for H. australis is achieved at 15°C water temperature (Kabir, 2001). The 21 week conditioning period for H. australis at 15°C (EAT = 1447 °C-days) identified by Moss (1998) is consistent to the findings of Kabir (2001) who found that the conditioning period suggested by Moss (1998) may be shortened to 19 weeks by raising water temperatures to 18°C for three weeks prior to spawning (calculated EAT = 1391°C-days). The 90 day (13 week) period of conditioning prior to
the spawning of *H. australis* found in this thesis research (calculated EAT \( \geq 448 – 898 \, ^{\circ}\text{C} – \text{days} \) at 10-15 \(^{\circ}\text{C}\) ambient water temperatures) suggests that the adult broodstock used in the spawning were well conditioned prior to the arrival at the hatchery.

Adult *H. australis* are very sensitive to changes in environmental conditions during the conditioning period. Changes in temperature, water flow rate, and food can all have an effect on the reproductive condition of broodstock and their ability to spawn (Moss, 1998). The stress response cause by handling and removal of the abalone from tanks to check the sex individuals and gonad condition may have had an effect on the success of the spawning.

**Recommendation for Future culture:** *Haliotis australis* broodstock should be segregated into separate sexes, bought into, and maintained in, and conditioned within ‘tipper’ tanks, using a constant water temperature of 15\(^{\circ}\text{C} \pm 0.5 \, ^{\circ}\text{C}\) within a controlled temperature environment that is maintained at 15\(^{\circ}\text{C}\). The abalone should be fed an ad-libitum diet of artificial abalone feed that has a high (~40\%) protein content which is supplemented by the addition of fresh seaweed (*Lessonia sp*. *Gracillaria sp*.). Under these recommended conditions, an EAT of \( \geq 1400 \, ^{\circ}\text{C} \, \text{days} \) (approximately 21 weeks) can be relied upon to ensure *Haliotis australis* are ripe and ready for spawning. The broodstock conditioning tanks should be configured so that the tanks can also be used as spawning tubs (into which spawning induction chemicals can safely added and removed) so that the abalone may be spawned in-situ without handling.

**4.3 Spawning and Larval Rearing**

Spawning of *Haliotis australis* in nature occurs after a change in seasonal water temperature (Poore, 1972b; Wilson, 1987). *H. australis* may spawn twice a year in the wild, but sometimes they may not spawn at all (Poore, 1972b). Elevating water temperatures was found to increase the numbers of adults that could be induced to spawn and produced a more rapid initiation of spawning when exposed to stimulus (Moss, 1998). Higher temperatures hasten the final stages of maturation of the gonad in some abalone species (Uki & Kikuchi, 1984). Spawning of *H. australis* is generally low or non-existent in wild caught broodstock (Moss, 1998). Comparison of the egg quality and quantity during the spawning of wild caught broodstock and hatchery reared broodstock at Rainbow Abalone Ltd in November 1995 showed that hatchery reared broodstock were induced to spawn more readily and produced higher quality gametes than wild caught broodstock. The successful spawning of *Haliotis australis* during this research project, and research conducted by Moss (1998) show that conditioned adult *H. australis* can produce commercial quantities of larvae for settlement using a hydrogen peroxide technique for spawning induction (Morse et al., 1977) and larval rearing in flowing seawater (Tong et al., 1992). Moss et al. (1995) concluded that the chemical induction method using hydrogen peroxide was a more reliable method of inducing New Zealand abalone species to spawn than the U.V. irradiated seawater methods (Seki, 1980, Uki & Kikuchi, 1984) used to spawn abalone elsewhere. Roberts and Watts
(2010) also had success producing larvae for research and small scale hatchery trials using the hydrogen peroxide technique to spawn *Haliotis australis*.

**Recommendation for Future Culture:** The slow elevation of seawater temperatures from 15°C to 18°C at a rate of approximately 0.5°C per day during the week prior to spawning and the use of the hydrogen peroxide technique is an effective method for induction of spawning in *Haliotis australis*.

### 4.4 Settlement and Settlement Cues

*Haliotis australis* larvae show the ability to test the substrate and to swim off it again, leading to asynchronous settlement (Moss 1999). Kabir (2001) noted that temperature has a profound influence on the physiology of *H. australis* and determined that the BZP for larval development in *H. australis* was 5.02°C which was identical to the BZP for gonad development). Roberts and Watts (2010) calculated the EAT (°C – hours) for larval development of *H. australis* (Table 6) using the following formula: water temperature of the larval rearing system (°C) minus the biological zero point multiplied by the age of the larvae in hours.

**TABLE 6: DEVELOPMENTAL INDICATORS IN Haliotis australis LARVAE OF INCREASING AGE.**

<table>
<thead>
<tr>
<th>Age (day)</th>
<th>EAT (°C – hours)</th>
<th>No. Rows of Radula teeth</th>
<th>No. of Cephalic Tentacle Buds</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1,138</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>6</td>
<td>1,365</td>
<td>0</td>
<td>0-2</td>
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<tr>
<td>7</td>
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<td>6</td>
<td>4-5</td>
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<td>10</td>
<td>2,287</td>
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<td>4-5</td>
</tr>
<tr>
<td>11</td>
<td>2,527</td>
<td>8-9</td>
<td>5-6</td>
</tr>
</tbody>
</table>

EAT, effective Accumulated Temperature (Seki & Kan-no, 1981b)

The *H. australis* larvae used in the experimental settlement for this thesis research were determined to be competent to settle at ten days development at ambient seawater temperatures of 15°C to 16.5°C (calculated EAT of 2155 - 2450°C – hours) and were observed to have 10 rows of radula teeth and four cephalic tentacle buds present. The observed development of the radula was slightly more advanced than the stages of development identified in Table 6 (Roberts & Watts, 2010) and the variation can be accounted for by likely fluctuations of temperature within the larval rearing tanks as a result of water temperatures within the experimental tanks following ambient air temperatures during the day as a result of the hatchery / larval rearing facilities not being insulated or temperature controlled.

Generally late stage abalone larvae tend to show higher rates of settlement and metamorphosis than early larvae (Seki & Kan-no 1981a; Moss & Tong, 1992a). Late stage larvae have more advanced radula development and delaying larval settlement should ensure that when larvae come into contact with a suitable substrate they are ready to settle and start feeding (Moss & Tong, 1992a). However while late stage *Haliotis australis* attach and crawl on settlement surfaces a high proportion retain a swimming and searching behavior (Moss, 1999).

**4.4.1 Diatom Biofilms as a Settlement Cue.**

Diatom biofilms are often used as surfaces on which to settle larval abalone (Roberts, 2001a). However during larval development abalone larvae may absorb dissolved organic material as a source of nutrition (Manahan & Jaekle, 1992) and newly settled larvae are able to utilise extra-cellular secretions from algae as their initial source of nutrition (Kawamura & Takami, 1995; Daume et al., 2000; Kitting & Morse 1997). Late stage swimming larvae may also ingest organic material or bacteria from the biofilm to remain alive while they search for a suitable substrate (Roberts, 2001a). Diatom biofilms that used in hatcheries as a settlement substrate are often conditioned using filtered seawater and an inoculation of a diatom slurry that has been filtered to retain only diatoms of a suitable size for ingestion by larval abalone (Roberts, 2001a). Inoculation of settlement tanks with a diatom slurry filtered to 5µm and conditioned for eight days with 1µm filtered seawater was the method adopted in the preparation of diatom films for this study. Moss (1999) confirmed the ability for *H. australis* larvae to feed prior to metamorphosis as evidenced by benthic diatoms being found in the stomachs of swimming larvae and attached larvae that had not yet metamorphosed after seven days of being introduced into settlement tanks when larvae had developed 15-17 rows of radula teeth. However use of diatom biofilms alone may not be sufficient to induce metamorphosis in cultures of *H. australis*. Roberts and Watts (2010)
identified that *H. australis* showed a relatively strong attachment response when exposed to diatom biofilms but showed low (<20%) metamorphosis of the attached larvae after four days.

The large numbers of swimming larvae that were observed in the experimental ‘No Biofilm’ and ‘1 Day Biofilm’ Replicates at 4-days post settlement, and which were subsequently removed from the experimental systems (as they had not settled by 4 days post settlement when, the banjo sieves were removed from the tank outflows), were found to have 14 rows of radula teeth and were likely to have been ready to attach and metamorphose should there have been a suitable substrate present on which to settle. The inferred settlement results in this study indicated that diatom biofilms alone had low settlement success and were statistically lower that

**Recommendation for Future Culture:** That the introduction of the veliger larval *Haliotis australis* into settlement tanks be delayed until at least six buds on the cephalic tentacles are visible and 9-10 rows of radula teeth are present in the veliger larvae (approx. 11-12 days at 15°C or EAT ≥ 2635-2870°C – hours).

**4.4.2 Pre-grazing to produce mucus trails as a settlement cue.**

Pre-grazed ‘8 Day Grazed Biofilm’ experimental tanks produced the highest inferred settlements at 33-days post settlement and post larval survival in the research conducted for this thesis. The removal of large numbers of the shells from post larvae from the pre-grazed ‘8 Day Grazed Biofilm’ replicates during cleaning operations up to 33-days post settlement suggest that initial settlement and metamorphosis of *H. australis* was initially high. A comprehensive review of abalone settlement cues by Roberts (2001a) documents the widespread use of pre-grazing of diatom biofilms as a method for inducing abalone larvae to settle in commercial culture settings. The generation of mucus trails by conspecific adults on settlement surfaces may be an effective settlement inducer a number of reasons: Pre-grazing may remove the loose over story of diatoms from the settlement surface leaving the prostrate diatom types that are most suitable for settlement (Mathews & Cooke, 1995); the pedal mucus may provide a nutrient medium to encourage the growth of diatoms and bacteria suitable for ingestion by post-larvae (Austin et al., 1990); and the mucus trails may trap diatoms on its adhesive surface (Davies et al., 1992) and the pedal mucus may become studded with organic material which in turn may be ingested by settling larvae. The mucus may also act as a fertilizer for microbial growth (Davies & Hawkins, 1998) and may contain high energy muco-polysaccharides that can be utilized a source of nutrition for early post larvae (Cully & Sherman (1985).

Laimek et al. (2008) identified that GABA was present in the nerves and epithelial cells of the foot, including mucus of several species of *Haliotis* and that the mean concentration of GABA in samples of dried pedal mucus averaged 0.68 mM GABA which suggests that GABA within the pedal mucus may
have a role in the induction of metamorphosis. Roberts and Watts (2010) reported that the *H. australis* has a species specific response to mucus trails with metamorphosis induction in *H. australis* doubled if larvae were settled on a diatom film pre-grazed by *H. australis* juveniles. Pre-grazing of diatom films by the non-conspecific abalone (*H. iris*) prior to settlement failed to enhance settlement of *H. australis* larvae in the same study. This suggests that the GABA present in abalone pedal mucus is not solely responsible for the induction of metamorphosis in *H. australis*. The same study noted that pre-grazing of diatom biofilms significantly reduced diatom densities within treatments and that densities of bacteria were lower in un-grazed treatments (Roberts & Watts, 2010) which further suggests a role of bacteria associated with the pedal mucus as having role in the induction of metamorphosis in *H. australis* larvae.

**Recommendations for Culture**: Pre-grazing with conspecifics appears to provide suitable cues for induction of metamorphosis, and produced the highest larval settlements in this study. However the usefulness of pre-grazing as a method for settling larvae in a commercial setting is challenging due to coordination of timing and intensive labor involved with handling of adults / juveniles required to prepare the pre-grazed diatom biofilm surface.

### 4.4.3 GABA treatments as a settlement Cue

Larvae treated with the settlement inducer GABA, showed a strong initial settlement response in this research. Moss (1999) also found that GABA hastened, but did not improve attachment and metamorphosis. Akashige *et al.* (1981) suggested that GABA narcotises the velar cilia of swimming larvae which caused veliger’s to “fall out of solution” to lie on the substrate. The use of GABA as a settlement cue has been systematically reviewed by Roberts (2001a) who reported that many commercial hatcheries initially discouraged the use of GABA due to poor results in the absence of antibiotics (Morse et al. 1979; Slattery, 1992) and concerns about toxicity on larvae due to prolonged exposure of high concentrations of GABA (Morse et al. 1980; Akashige *et al.*, 1981). However the effective use of GABA without antibiotics in the presence of a diatom biofilms has been demonstrated successfully in large scale induction of metamorphosis in *H. rufescens* in a commercial context (Searcy-Bernal *et al.*, 1992b). GABA has been identified as being optimally active as a settlement cue at concentrations of ~1µM for a range of *Haliotis* species (Roberts, 2001a). *H. australis* larvae shows a dose related response to GABA treatments with metamorphosis observed to peak in concentrations of 1µM GABA while declining at higher and lower doses (Roberts and Watts, 2010). The same study found that attachment of *H. australis* larvae was high (~80%) when subjected to a treatment of 1µM GABA with moderate metamorphosis (55%) having occurred after 4 days, and bioassays containing combination of GABA treatment and diatom biofilms pre-grazed with conspecifics have been shown to induce high levels of metamorphosis (81%) and post larval shell growth (>60%) after 4 days (Roberts and Watts, 2010).
Competent *H. australis* larvae in this thesis research were exposed to a 30 minute ‘dip’ of 1 mM GABA prior to introduction into the settlement tanks and appeared to show a strong initial settlement response within all treatments, however this was not statistically significant at a 5% level of confidence. After several days many of the larvae were observed swimming, particularly in tanks with no established diatom biofilm present which may have resulted from the initial narcotisation of the velum wearing off and a reduction in the effect of GABA on the larvae as a result of dilution and dispersion of the active chemical by seawater. GABA is highly susceptible to degradation by marine microbes (Caspar & Mountfort, 1995) and a wide range of bacteria can utilize GABA as a source of carbon, nitrogen and energy (Kaspar et al., 1991). This susceptibility of GABA to degradation should ease concerns around toxicity of GABA solutions used to settle larvae, particularly when larvae are exposed to relatively low doses of GABA (1 µM) and it is likely that rapid bacterial degradation of the GABA solution reduces the potential for toxic effects on the development of post larvae (Searcy-Bernal and Anguiano-Beltran 1998). GABA concentration can be doubled or tripled without compromising metamorphosis or post-larval survival in *Haliotis rufescens* (Searcy-Bernal and Anguiano-Beltran, 1999). It is possible that the bacteria utilising GABA as a food source may in turn be a food source for swimming, attached and metamorphosed larvae.

**Recommendations for Culture:** Immersion of competent larvae in a 1 mM dip of GABA prior to introduction to the settlement tanks may encourage competent larvae of *H. australis* to quickly make contact with and attach to the substrate, rather than remain swimming (as was observed in the absence of GABA). The initial seawater water in tanks used to settle *H. australis* larvae should contain an initial treatment of GABA up to 1-2 µM GABA that is held within the settlement tank for several days before water flow is restored. The use of GABA should be considered to encourage the *H. australis* larvae to settle particularly in the absence of pre-grazing of settlement tanks by conspecifics. The asynchronous settlement of *H. australis* larvae is likely to remain one of the biggest problems for those wanting to culture *H. australis* until suitable biofilms have been identified.

**4.5 Post larval Development and Survival**

High mortality in abalone larva is a widespread problem in abalone hatcheries around the world (Ebert & Houk, 1984, 1989; Hahn, 1989; Ebert, 1991) with *Haliotis australis* continuing to provide challenges to culture due to high mortalities near or and just after settlement (Moss, 1998; Naylor & McShane, 1997; Roberts & Watts, 2010). This research identified that major periods of mortality occurred immediately after settlement and again between 14 to 21 days post settlement. As previously indicated in mortalities from 14 days post settlement to 21 days post settlement may have been due to ciliate infestations, or from starvation from being trapped amongst a rapidly developing diatom biofilm, or exhaustion from insufficient nutrition during energy expensive periods of growth (when the peristomial shell overlaps the larval shell for the first time). Care must be taken by culturist to ensure periphyton
and biofilms are managed to ensure that the post larvae have access to diatoms or alternative food sources that they can ingest. Post larval abalone with a shell length of 600 - 800 µm become responsive to ‘digestibility’ of diatoms cell diets and grow more rapidly on efficiently digested diatom species and seem to require access to diatom cell contents for rapid growth (Kawamura et al., 2005). As abalone grow it becomes increasingly difficult for culturists to maintain adequate microalgae food supplies, and supplement diatoms or other food sources are required to be added to culture tanks. In some hatcheries post larvae >1 mm shell length are slowly weaned onto small amounts of powdered artificial diets so that by 3mm shell length they are feeding completely on manufactured feed (Kawamura et al., 2005). This would suggest that attempts to supplement the diet of *Haliotis australis* with powdered artificial feed could have been attempted from ~30 days post settlement in this study.

Ciliates were associated with high mortalities of larvae in all treatments seen in this study and similar effects of ciliates were observed in small scale hatchery trials of the settlement of *Haliotis australis* by (Roberts and Watts, 2010). A high degree of environmental control can be achieved with bioassay experiments but control of parasites and predators is much more difficult in large scale experimental / trial systems.

Large numbers of healthy *Haliotis australis* juveniles were observed growing in the PVC drainage gutters that channeled waste water from the experimental tanks in this study. These animals continued growing vigorously throughout the study and were the only animals still alive 1-2 months after the completion of the experiments. These juveniles may have come from a settlement of swimming larvae that were discarded into the drains at the first drain and fill or possibly as a result of larvae that may have been flushed out of the tanks on clumps of diatoms. The shallow (<5 mm deep) flowing water and vigorous diatom growth within the drains appeared to suit *H. australis* particularly once they had reached 2-3 mm in length. It may be that these conditions made it more difficult for predators and competitors such as ciliates and copepods to flourish as was seen in the deep settlement tanks that had relatively static conditions with slow water inflows and outflow of water.
5.0 Conclusions.

5.1 Final Overview of Research into the Culture of Yellowfoot Paua

This research was conducted at a time when New Zealand abalone culturists had established methods for culture of the Blackfoot Paua *Haliotis iris* (Tong et al., 1992) and there was considerable interest in the development of an alternative native New Zealand species of abalone for aquaculture. There was significant industry interest in conducting experiments on the Yellowfoot Paua *Haliotis australis*, to determine if commercial scale culture methods for *H. iris* could be crossed over and applied to *H. australis*.

The Graduate Research in Industry Fellowship (GRIF) project, on *H. australis*, sought to establish if standard methods used to culture of *Haliotis iris* were indeed applicable for the culture of the Yellowfoot Paua. The time frame of the project allowed for only a single spawning, and subsequent culture and settlement of *H. australis* larvae. However results of this experiment confirmed that methods for the spawning and raising of *H. iris* larvae can be used to successfully produce viable gametes and competent larvae of *H. australis*.

Settlement of *Haliotis australis* has often been identified as the most critical and challenging period in the culture of Yellowfoot Paua. The asynchronous settlement behavior of *H. australis* larvae has significant implications for the successful aquaculture of this species. The GRIF project conducted at Rainbow Abalone Ltd in 1995 can be best described as a pilot hatchery scale project which sought to duplicate and affirm the initial findings of the work on *H. australis* undertaken by Mr. Graeme Moss in the early 1990’s. The research has established that larval settlement was generally low and post larval survival was low in most of the experimental treatments with pre-grazed diatom biofilms and a combination of diatom biofilms and GABA treatments showing the most promise for use for a commercial scale production of *H. australis*. However the results of the experiments indicate that settlement surfaces used in the experiments may not yield commercially viable numbers of post larvae for production of this species. The settlement experiments yielded a statistically significant result identifying the most successful settlement surfaces and treatments for settling *H. australis* larvae. However a cautious approach should be taken when drawing firm conclusions as settlement success in each treatment was inferred from survival data at 33 days post settlement. Observations of periods of high mortality prior to the 33 day post settlement census date serve as an indicators of important periods when in the culture of the Yellowfoot Paua when factors such as density of settlement and food availability may have an influence on the survival of settled larvae. These mark important periods on which to focus in future research on this species. The GRIF research determined that the highly sensitive and mobile Yellowfoot Paua is a very difficult species to culture.
Arguably the most significant contribution from this research to the body of knowledge surrounding *H. australis* was the production of the SEM micrographs of the developmental stages of *H. australis*. Copies of the photomicrographs were distributed (in 1996) to key researchers working on abalone in New Zealand and it was the hope of the author that they were of some interest and value for their research. In 1995 much of the information on the culture of Yellowfoot Paua was anecdotal, and other than the published work by Poore (1972) and Wilson (1987) on the ecology of *H. australis* in the wild, there was little if any published material on the aquaculture of *Haliotis australis*. In the 20 years since the original experiments were undertaken for this research there has been an extensive development of the knowledge abalone culture in general, and a large body of published research has developed.

Significant and systematic research has since been conducted and published on *Haliotis australis*. The early research undertaken research in the 1990’s by Moss focusing on the general culture of Yellowfoot Paua, the conditioning of broodstock and examination on factors affecting settlement were published in the late 1990’s (Moss, 1997; 1998; 1999). Roberts (2001b) has made significant contribution to the general body of knowledge of larval settlement and post larval performance in New Zealand *Haliotis* species, and has compiled a comprehensive review the published research from around the world into settlement cues affecting larval settlement and metamorphosis (Roberts (2001a). Determining the biological zero point for both gonad and larval development for *Haliotis australis* by Kabir (2001) has enabled the calculation of an EAT for spawning readiness and is of significant practical use for those that wish to culture Yellowfoot Paua. More recent detailed investigation into the settlement preferences of *Haliotis australis* by Roberts and Watts (2010) have provided additional evidence and robust statistical analysis, that build on and support the findings of Moss (1999) and also on the experiments and observations undertaken in 1995 as part of this research.

5.2 Areas of Interest for Future Research

With respect to *Haliotis australis*, future research is recommended on the development of settlement surfaces which improve larval settlement. The development of CCA encrusted settlement plates combined with diatom biofilms (which were observed in use by Brown at Crystal Park Marine Farm), (pers. obs.1995) may produce more reliable settlements of *H. australis* when compared with traditional settlement surfaces.

Development of a water stable, powdered, artificial or natural (seaweed) weaning diet for post larva suitable to be used as a supplement to, or total replacement for, diatom food sources at approximately four weeks post settlement is required.

Suitable tank design for the cultivation or larval and juvenile *H. australis* is an area of future research interest. The highly mobiles juveniles of *H. australis* were often found outside of the deep slow flow
cultivation tanks and the new generation tipper tanks and trays used in the grow out of *H. iris* may be suitable for cultivation of *H. australis*. Observations of successful growth and survival of juvenile *H. australis* that had ‘escaped’ into the PVC guttering removing wastewater from the experimental system, suggest that a shallow, high flow rate tank design may be suitable for post larval growth this species.

### 5.3 Final Comments

In 2014 a significant body of knowledge on the culture of *Haliotis australis* has been developed that suggest that commercial production of this species is possible in the long term. The research to date suggests that asynchronous larval settlement and high larval mortality at settlement remain major challenges. Post larval culture may also have particular challenges that are not yet well understood which may be exacerbated to the highly mobile behavior of juveniles. Any commercial venture seeking to undertake production of Yellowfoot Paua in New Zealand as a sole basis for an abalone business will be a very high risk venture, which is unlikely to be economically viable on its own account and should be approached cautiously. That said, *Haliotis australis* will continue to be of interest as a candidate for further future aquaculture research and development and is most likely to be furthered by an established commercial (*H. iris*) hatchery as an alternative niche product.

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6.0 References


Gmelin, J. F. (1791). Caroli a Linné. Systema Naturae per regna tria naturae, secundum classes, ordines,


