Dietary Protein Requirements of the New Zealand Black-footed Abalone (*Haliotis iris*, Martyn 1784)

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Attestation of Authorship

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

Signed……

Date……

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Abstract

The aim of this thesis is to evaluate the nutritional aspects of dietary protein for the New Zealand black-footed abalone (*Haliotis iris*) as an aquaculture species, and thus provide an affordable option for formulated feeds to be used in this abalone industry.

Young (4 hours) and mature (7 days) microalgal biofilms were tested for their effectiveness on *H. iris* larval early developmental processes (attachment, metamorphosis, settlement and survival). Amino acid profiles and percent biofilm cover of microalgae also were monitored. Significantly better effects on attachment, metamorphosis, settlement and survival were found when abalone were exposed to mature microalgal biofilms compared to young biofilms, but few significant differences were found among biofilms composed of different microalgal species. Attachment and metamorphosis did not appear to be affected by differences in essential or non-essential amino acid profiles in the diets, but positive correlations were found between developmental processes and total amino acid content and percent biofilm cover.

Out of the twenty microalgal strains tested, nine of those strains were mixed to produce dual microalgal biofilms, and their effect on the settlement of *H. iris* larvae was monitored. As with the single microalgal biofim results, attachment and metamorphosis showed a positive correlation with total amino acid contents in the biofilms. Amino acid profiles also did not correlate well with attachment and metamorphosis in the present experiment. These results indicate that larval settlement was significantly affected by protein content (by means of total amino acid contents in biofilms) compared to the effect of different microalgal species or amino acid profiles in the biofilms.

Nine commercially available protein sources (white fish, red fish, blood, meat and bone, casein, soybean, gluten, maize protein, *Spirulina*) were used as sole protein sources in formulated diets which contained 33% crude protein, 5% lipid and 40% starch to feed juvenile *H. iris* at 18 °C for five months. Juvenile abalone fed with diets containing white fish, red fish, casein, soybean and *Spirulina* had significantly better growth than those of fed other protein sources, thus indicating that these diets could be suitable as sole protein sources for *H. iris* formulated feeds. Abalone juveniles fed soybean diets (with extremely low methionine

content) had similar growth results as those fed fish meal diets, and did not show a decrease in certain amino acid content (such as methionine) in their soft body tissues. This result was interpreted as a sign that the methionine requirement is low for *H. iris* juveniles. However, dietary amino acid profiles did affect the amino acid profiles of both soft bodies and shells, which may have caused changes in shell bio-mineralization.

Based on the overall growth and shell morphology parameters which were monitored, red fish protein had the best performance and it was therefore used to determine protein requirements under two different temperature regimes (13-21°C and 8-16°C) that simulated the temperature regimes of the South and North Islands in New Zealand. Six different dietary protein levels (0, 10, 20, 30, 40, and 45%) made from red fish meal were fed to juvenile abalone for four months. Growth performance increased steadily with increasing dietary protein. Similar growth results were observed under two temperature regimes when dietary protein levels were above 40%. However, a second-order polynomial curve fit showed differences in protein requirements between the two temperature regimes. In other words, animals maintained under low temperatures required more protein (42-53%) than those in high temperature regimes (38-39%). However, protein levels did not appear to affect amino acid profiles of both soft body tissues and shell material within either temperature regime.

Shell morphology of previous two dietary experiments (nine different protein sources and six different protein levels under two temperature regimes) was analyzed at the end of each experiment. Shell length, width, height, thickness and weight were monitored. Dietary protein sources and levels strongly affected *H. iris* shell morphology. Significantly wider, higher and heavier shells were found in animals fed casein diets. Both fish meal diets resulted in abalone with flatter and heavier shells. High dietary protein levels also promoted the enhancement of shell width and shell weight, and high temperature environments produced heavier and thicker shells.

In conclusion, dietary protein is an important factor for *H. iris* aquaculture. The content of dietary protein strongly affects settlement of larvae and growth of juveniles in *H. iris*. Dietary amino acid profiles can change amino acid profiles in both soft bodies and shells, which may cause changes in shell morphology. A dietary protein content above 40%, as can be provided with red fish meal in formulated diets, is recommended to improve *H. iris*

production. Raising dietary protein contents can effectively recover the decreased growth that results from a low cultivation water temperature regime.

Chaptet 1. Abalone Ecology and Aquaculture

1.1 General Biology and Ecology

1.1.1 Taxonomy

The word "Abalone" is derived from the American English variant of the Spanish name "Abulón", used for various species of single-shelled mollusks from the *Haliotidae* family (genus *Haliotis*) whithin the Class *Gastropoda*, and the Phylum Mollusca. Linnaeus (1758), first described this species, and depicted the first seven species of abalones (Geiger, 1999). The generic name *Haliotis*, meaning "sea ear", was given by Linnaeus in 1758.

1.1.2 Morphology

The family has unmistakable characteristics including a rounded to oval shell, two to three whorls, and the last auriform one that will grow into a large 'ear', thus deserving the moniker as "ear-shell." The body whorl has a series of holes (four to ten depending on the species) near the anterior margin (Hahn, 1989a). Its single shell is conspicuously convex, ranging from highly arched to very flat. The shell is tightly connected to the body by shell muscles. A big muscular foot enables it to adhere to rocky surfaces at a variety of sub-tidal depths around the coast.

The internal and external shell's colors vary greatly from species to species, and so does the inside of shell. No matter what species, the inside of their shells can radiate iridescent, glittering colors, ranging from silvery white to green and red. The New Zealand black-footed abalone (*Haliotis iris*) exhibits pink, purple, green and red with a predominant deep blue (Bevelander, 1988; Hahn, 1989a).

Their shells are ear-shaped and have a small spire standing in the anterior region. The shells of most abalones constitute a large whorl with an enormous aperture. A prominent feature on the dorsal surface is the presence of respiratory pores. Interestingly, excretory

products, as well as sperm and eggs, ooze their way through these holes into the respiratory current that enters at the front of the shell and flowing over the gills (Tong, 1992).

1.1.3 Distribution

Members of the family *Haliotidae* flourish in rocky shores of most tropical and temperate oceans, particularly in shallow sub-tidal zones. The Haliotidae are widely found in the coastal areas all over the world. Most species are encountered in shallow sub-tidal depths between 10 m and 30 m sea levels. Their sizes are quite different, depending on species, and can vary from 20 mm (*Haliotis pulcherrima*) to 200 mm or even more, such as in *H. rufescens* (Hahn, 1989a).

Most species have localized distributions. However, Stewart and Geiger (1999) reported three Indo-Pacific taxa with a wider range of distribution—*H. clathrata,* from East Africa to Samoa; *H. asinina* and *H. planate*, from Thailand to Fiji; and *H. ovina* (from Maldives to Tonga). Other species are all confined to much more restricted ranges. Five endemic species have been known to thrive in South Africa, three in New Zealand, six on the western North American coast, and nine in Australia (Geiger, 1999).

1.1.4 Haliotidae in New Zealand

There are three recognized species of abalone endemic to New Zealand and its surrounding islands. The black-footed abalone (*H. iris*; Martyn 1784) is the main commercial species (Dutton and Tong, 1981), while its smaller relative, the queen or yellow-foot abalone (*H. australis*; Gmelin, 1790) is also harvested commercially (O'Halloran, 1986). A third species, the virgin or white-footed abalone (*H. virginea*; Gmelin, 1790) has four geographically separated subspecies--*H. virginea virginea*, *H. virginea crispata* (Gould, 1847), *H. virgina huttoni* (Filhol, 1880), and *H. virginea morioria* (Kaicher, 1981; Ubaldi, 1986), but they are not harvested commercially due to their small size and low abundance. Comparatively, *H. iris* can reach a size of up to 195 mm in shell length, *H. australis* to 110 mm, but *H. virginea* to only 70 mm (Dutton and Tong, 1986). However, the lifespan of all three species can reach more than ten years (Dutton and Tong, 1981).

The black-footed abalone, *H. iris*, is also called "pāua" in New Zealand, which is an native name of New Zealand Maori. This species of abalone has relentlessly been harvested by Maori for hundreds of years and they are discontinuously distributed along the coasts of both main islands of New Zealand and the Chatham Islands, Stewart Island, and the Snares Islands (Sainsbury, 1982). Their population straddles more than 20 degrees of latitude (McShane et al., 1994), but they are more abundant from the Wairarapa Coast southward (Elvy et al., 1997). *H. iris,* a shallow-water species, lives in both sheltered and exposed shores. It is habitually most abundant at < 5 m depth, but its distribution may extend to 20 m in depth (Sainsbury, 1982, Scheil et al., 1991). Pāua are found in both sub-tidal and sub-littoral fringes, inhabiting rocky substrates, particularly flat boulder bottoms, or low rocky ridges (Poore, 1972a). All abalone species prefer sites where there is well oxygenated sea water and where salinity is stable (Hahn, 1989c).

1.2 Reproductive biology

Abalones reach maturity at a relatively younger age and with smaller maturation body size in comparison with fish. Nevertheless, their fertility is exuberant and normally increases

with size (from 10,000 to 11 million eggs at a time) (Hahn, 1989d). Many studies have been conducted on the reproductive biology of various abalone species (Webber and Glese, 1969; Young and DeMartini, 1970; Poore, 1972c). Crofts (1929) found that the gonad, without any accessory glands, eventually develops a layer covering a cone-shaped digestive gland in both sexes. Its colour is most easily distinguished when the abalone is sexually mature.

Previous investigations into the reproductive biology of the New Zealand black-footed abalone, *H. iris*, were conducted by Poore (1972c), Sainsbury (1982), and Kabir (2001). From these studies it is known that *H. iris* in the North Island spawn in winter, while those in the South Island do so during summer and autumn. In contrast, the *H. australis* populating both islands spawn only during summer and autumn (Poore 1972c; Kabir, 2001). Poore (1972c) even attempted to correlate changes in sea temperature with gonad maturation and spawning, and he found a slight correlation in two above-mentioned species in one particular year but not in the following year. Later, McShane and Naylor (1996) reported a positive correlation between change in gonad index and sea temperature which was calculated by the way of summation of the daily exposure to the temperature above biological zero point (BZP).

Abalone are dioeciously broadcast spawners. Male and female abalones release thousands of sperm and eggs directly into the surrounding seawater through the holes in their shells (Shaw, 1991). Sudden temperature changes, exposure to air and release of gametes by other abalones are thought to trigger gravid abalone spawning. External fertilization (Shepherd, 1976) is followed by the development of a lecithotrophic larval stage (Leighton, 1974). The pelagic nature of this phase of the life cycle is thought to facilitate their dispersal (Prince et al., 1987). The larvae may spend from 2 to 14 days in the water column (Leighton, 1974; Glover and Olsen, 1985).

Within a day of fertilization, eggs can develop into trochophore larvae and they in turn can develop into veliger larvae over the following days. The swimming stage for most abalone species can last several days. It generally takes *H. iris* larvae seven days to develop at 15~20 °C. The length of this stage may vary under different temperatures, and at this stage, larvae do not take any food, but consume the nutrients from the yolk (Hahn, 1989b).

1.2.1 Larval settlement

Settlement is a transition from swimming larvae to post-larvae, crawling on the bottom of the ocean, and starting to develop a shell. The whole settlement processes can be divided into three stages (as described by Roberts 2001b**)**. Once settled, abalone assume a benthic existence for the remainder of their life cycle (Wood and Buxton, 1996). Settlement and recruitment of abalone generally occurs on coralline-encrusted rocks from the low tide mark to several meters in water depth.

The first stage is called *attachment,* during which the larvae stop swimming, sink to the bottom, and attach to the substratum with their feet. Attachment is a reversible behavior. At this stage, the larvae display "searching behavior" which allows them to test the substratum quality and enables them to choose and move to another place.

The next stage is characterized by the initiation of *metamorphosis,* which is marked by shedding larval vela, and by committing abalone to benthic life. This process is accompanied by the formation of mucus glands, blood circulation, digestive tubes and mouths. Instead of consuming energy in yolk, feeding and ingesting nutrients from the environment begin at this stage and flared peristomal shells will thus start to grow.

The last stage of metamorphic development is referred to as *settlement*. Instead of flared peristomal shell growth, the shell growth becomes spiral and changes in line with the horizontal axis direction.

The terms for these three stages will be used when describing the transition from a larva to a post-larva in this study. Generally speaking, the post-larva is defined as a period which begins with attachment and lasts until the first respiratory pore appearing in the shell. In the case of *H. iris,* its body generally grows to 2 mm in length at the age of two months (Kawanura et.al., 1998). Once the abalone develop respiratory pores at about 2 mm in length, their shells become more and more hardy, which results in their survival rates being relatively high and predictable in an aquaculture system (Fallu, 1991).

Settlement was found to be affected by chemical stimuli which emanate from various substrates or natural biofilms. For example, chemical stimuli from mucous trails of grazing individuals have been shown to induce con-specific settlement under culture conditions (Seki and Kanno, 1981; Barlow, 1990; Slattery, 1992). The extract gamma-aminobutyric acid, obtained from encrusting coralline algae (CCA) such as *Mesophyllum discrepans,* has been shown to be responsible for inducing settlement (Morse et al., 1979; Morse and Morse, 1984b; Shaw, 1991). Laboratory studies of monospecific biofilms and isolated chemicals, such as gamma-aminobutyric acid (GABA) and potassium chloride, have highlighted the importance of a range of chemicals for settlement, which may be produced by different biofilms (Morse and Morse, 1984a; Morse et al., 1984; Bryan and Qian, 1997; Roberts, 2001a; Lam et al., 2005). Cultured biofilms become the most common way to settle various marine invertebrates' larvae (including abalone) in both laboratory studies and commercial hatcheries (Hahn, 1989d). Recent studies have demonstrated that mono-specific bacteria or diatom biofilms can induce larval metamorphosis and settlement in bryozoans (Dahms et al., 2004), polychaetes (Unabia and Hadfield, 1999), mussels (Satuito et al., 1997), oysters (Fitt et al., 2005), barnacles (Maki et al., 1990), sea urchins (Huggett et al., 2006), and abalone (Roberts, 2001b).

1.3 Abalone Fisheries

1.3.1 World fisheries

Abalone harvesting practices were first reported by Aristotle in 4th century B.C. In the 1st century A.D. the name of *otia* (little ear) was used by Pliny. In Japan abalones were mentioned as early as the 4th century A.D. In the medieval literature of Europe they were alluded to for the first time by Gessner in 1553 (Geiger, 2000). The commercial fishery for abalones in California began in the 1850's. The flesh of *H. rufescens, H. carcharoides, H. sorenseni and H. fulgens* was canned or dried and exported to Asia (Hahn, 1989a). Customary harvesting of abalones by the native Maori people of New Zealand has been documented as far back as 1150 AD, and a substantial recreational fishery persists even to this day.

Currently, there are about 20 species of abalone harvested commercially worldwide, including one New Zealand species (Hahn, 1989a). The major abalone fishing countries are Japan, Australia, New Zealand, South Africa, Mexico and the United States (Gordon and Cook, 2001). Because of prolonged exploitation of these unsustainable resources, the world's stock of abalone has declined rapidly (Gordon and Cook, 2004). Abalones are extremely susceptible to over-fishing due to their slow growth rate, a tendency to aggregate in one area, and unpredictable recruitment (Tegner and Butler, 1985; Tegner, 2000). Because of their vulnerability to over-fishing, abalone fisheries around the world are heavily regulated by quota allocations, fishing gear restrictions, and seasonal fishing enclosure (Gordon and Cook, 2004).

1.3.2 New Zealand fishery

Abalone are found climging to rocks around the coast of New Zealand in shallow water. They are much more abundant, and grow much larger in colder waters around Southland and Stewart Island (Sainsbury, 1982). The three native species of abalones are easily distinguished by their shell morphology and colour. New Zealand Mäori have traditionally harvested and traded abalone for their flesh and their decorative shells. After shelling, the abalone flesh (the muscular foot) is eaten as a delicacy, while the shell is used for decorative

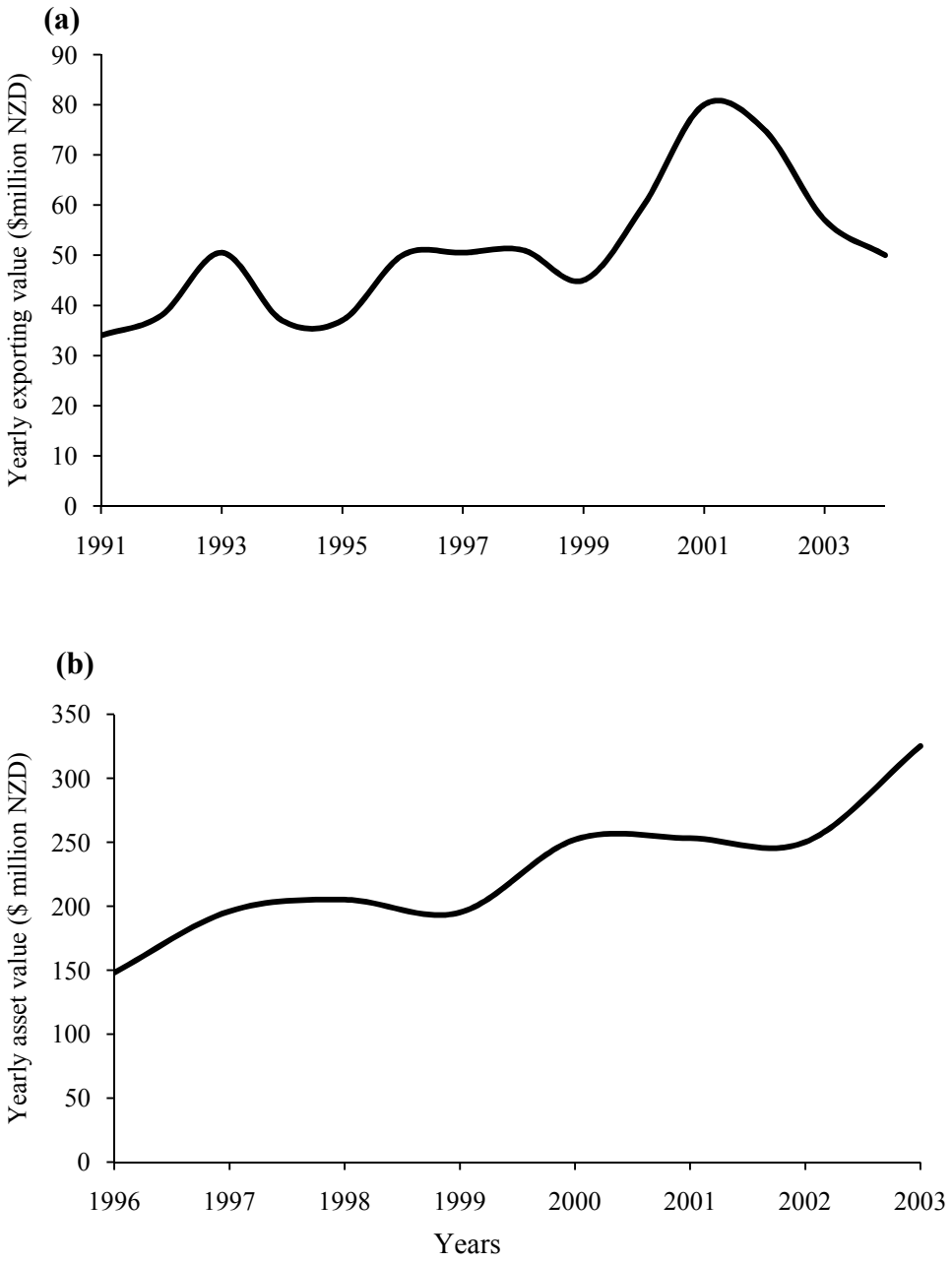
purposes. Abalone meat is exported and sold locally as both fresh and canned products. The abalone shell is known for its decorative colours and may be used to make jewelry, as embellishment on wood articles, stationery, and artist work, and as polished shells (Sainsbury, 1982).

The commercial fishery for abalone meat began in the 1960s in order to service a growing domestic market. During this period the fishery expanded rapidly due to the surge in demand. By the early 80's, considerable fishing pressure on the resource began building up, thereby prompting implementation of control measures which included moratoria of new fishing permits, restrictions on export quantities and length of fishing season (Elvy et al., 1997).

In New Zealand, abalone are legally caught under a limited catch per day by free divers (diving without any use of breathing apparatus) who use blunt instruments to prise shellfish from rocks. A large recreational fishery for abalones also exists throughout NZ. Two abalone species (*H. australis* and *H. iris*) were introduced into the Quota Management System (QMS) in 1986. The QMS monitors stocks and catches, allowing the government to set Total Allowable Catch (TAC) limits for individual species to ensure the sustainability of New Zealand's fisheries (Statistics New Zealand, 2004).

Abalone export revenues in New Zealand have soared from $34 million in 1991 to a peak of $80 million in 2001, but have since been skidding to $50 million in recent years due to a decline in resources (Figure 1.1a). Most of the exported abalone in 2004 was in cans or jars (89%), with the rest exported either chilled, dried, salted, in brine or live. Although the total catch of abalone has been decreasing year by year, the demand of overseas markets for abalone also has been surging yearly. This undoubtedly helps to boost the current asset values of the abalone industry. Under the QMS, rights to päua can be traded. When these rights are sold, the value for which they are sold may be used to calculate total asset values in the industry. The total value of New Zealand's päua was calculated to be around $330 million for 2003. The total value of abalone has increased steadily from $143 million in 1996 to $328 million in 2003, except 2000-2002 when the value remained relatively constant (Figure 1.1b).

Figure 1. 1: (a) New Zealand's abalone export value ($NZD) from 1991 to 2004. (b) The total asset value ($NZD) of the abalone industry from 1996 to 2003. These figures modified from Statistics New Zealand (2004). *New Zealand Official Yearbook 2004*, Statistics New Zealand, Wellington.



1.4 Abalone Aquaculture

There is good reason to argue that a rapid decline in wild-abalone fisheries has prompted increased practice of abalone aquaculture (Gordon and Cook, 2004, De Waal et al., 2001). Most of the world's abalone fisheries have declined over the past two decades and some, such as those in Mexico and California, have collapsed (Guzmán del Próo, 1992). Fisheries statistics indicate a global decline in abalone yield from 20,000 in 1975 to 13,600 tonnes in 1989 (FAO, 1975, 1989).

However, the major reason behind the rapid increase in abalone aquaculture is the lucrative market value for their large adductor muscle or foot (Elliott, 2000). The sedentary nature and shallow water habitat of abalones make them easily accessible to divers and, in the absence of strict control measures, they are vulnerable to overexploitation (Breen, 1992). The major markets for abalones are mostly in Asian countries, such as China, Japan and Hong Kong (Oakes and Ponte, 1996, Robertson-Anderson, 2003).

Current research on abalone culture has gained new currency in the academics of the world, but it was commenced by Japanese and Chinese scientists a few decades ago (Ino, 1952; Chen et al., 1977). The global production of abalone reached 22,600 metric tonnes in 2002, including poaching of 3700 metric tonnes. Of this, over 8,600 metric tonnes were farmed and their total value was estimated at approximately US$ 0.8 billion (Gordon and Cook, 2004). Triggered by a decline in yields from wild fisheries, a burgeoning cultivation of abalone was on the march in the 1990s. China is by far the largest producer in the world, with over 300 farms and a total production of approximately 4,500 metric tonnes (Gordon and Cook, 2004).

In New Zealand, owing to mainly harvasting abalone from beaches, more accessible sites have been over-fished to a point where there is localized depletion, with increasing pressure brought on abalone resources by the commercial, recreational, and illegal fishing. (Elvy et al., 1997; McShane, 1996; Sainsbury, 1982). This pressing situation has done much to boost abalone aquaculture. New Zealand also has an attractive location for aquaculture of many marine species thanks to its favourable environment and well-established seafood processing and marketing infrastructure (Harrington, 2000).

The first land-based abalone farm was established in 1987 (Roberts, 1999; Tong and Moss, 1992). Mahanga Bay Shellfish Hatchery in Wellington developed the commercial culture protocol for black-footed abalone, *H. iris* (Tong and Moss, 1992). There are around twenty-two abalone farms operating land-based systems with water pumped from the sea: twelve are in the South Island and Stewart Island, and ten in the North Island (Roberts, 1999). These farms aim mainly to produce a 'cocktail' abalone of 50-70 mm shell length. In recent years, the demand for cocktail-sized abalones in Asia has rapidly been growing and generating greater returns than the traditional market for wild-caught abalones (Oakes and Ponte, 1996). Considering it is illegal to harvest cocktail-sized wild abalones in New Zealand, aquaculture has been the only way to satisfy growing demand for these smaller animals.

Under the local government's waste discharge regulation, *H. iris* aquaculture in New Zealand has to come to grips with the only preferable culturing method, namely a land-based full- or semi-recirculation system. Even worse, most farmers use artificial diets for growing, partly due to restrictions on a harvesting of macroalgae drifting on the beach. In a recirculation system, farm operators need to control many factors to ensure the final success of abalone aquaculture. There are nutrient factors (protein, lipid, carbohydrate, vitamin and minerals) that directly affect the growth of the abalones in recirculation environment. In place of macroalgae, abalone farmers in New Zealand are obliged to use artificially formulated diets for better growth. The nutrient of a formulated diet has proved to improve growth for different abalone species in farm settings (Hahn, 1989b).

1.5 Nutrition

Abalone are herbivorous invertebrates and naturally feed on macroalgae (Sales and Britz, 2001; Nelson et al., 2002; Shpigel et al., 1999). Their preference varies worldwide, depending on their habitats and availability of macroalgal species (Nelson et al., 2002).

According to Poore (1972b), *H. australis* and *H. iris* preferred *Hymenocladia* (red algae) to *Lessonia* and *Macrocysis*. The latter species has been shown to grow faster when fed only

Hymenocladia (Poore, 1972b). In the wild, young *H. iris* juveniles generally inhabit the intertidal zone, preferring water less than 2 m in deep, where turbulent water and high levels of dissolved oxygen can be found (Fallu, 1991; Hahn, 1989a; McShane and Naylor, 1994). When pāua reach sexual maturity at approximately 70-90 mm shell length, they begin to migrate into deeper waters. Small abalone (less than 5 mm) graze microscopic algae, especially diatoms and ingest a range of bacteria (Garland et al., 1985). Larger juveniles and adults are more sedentary, feeding predominantly on drifting macroalgae by raising the front of their feet to catch drifting algae pieces (Poore, 1972a; Tong, 1992; Dutton and Tong, 1981).

The abalone natural feeding pattern is one of shifting to different foods at various stages of their life cycle (De Waal et al., 2003). That is attributed not only to the increased mouth size (Fleming et al. 1996), but also to morphological changes of the radula as the abalone grow (Kawamura et al., 2001; Daume and Ryan, 2004; Onitsuka et al., 2004). Changes in radular structure and organization in *H. iri*s and *H. discus hannai* have been shown to prepare the animals for the feeding transition from micro- to macroalgae (Roberts et al., 1999; Kawamura et al., 2001).

As abalone grow, changes in diet could also take place due to transformations in the gut's micro-flora, such as bacteria and transformations of enzymes within the digestive system, which enable them to digest macroalgae (Erasmus et al., 1997; Tanaka et al., 2003). Stuart and Brown (1994) suggested that a variety of algae are better able to meet the preferences and nutritional requirements of cultured abalone over extended periods. From the perspective of an aquaculture operator, macroalgae are more difficult to obtain, to process for feeding, and to clean and store. In comparison with artificial pellets, macroalgae are more difficult to use and sustain the current commercial production of abalone (Hecht and Britz, 1990; Johnston et al., 2005).

Ogino and Ohta (1963), who were the first pioneers in abalone pellet diets, blazed a new trail toward a better understanding of various protein sources and optimal levels in an artificial diet for Japanese abalone. They found that using casein and soybean meal produced the highest growth rates in culturing *H. discus hannai*. Ogino and Kato (1964) took a big step by using fishmeal in test diets containing between 15 and 44% crude protein levels and

reported a linear relationship with the growth rate and protein level. In abalone aquaculture, farmers generally use microalgal bio-films to feed swimming larvae and young juveniles and then wean them onto kelp or formulated pellets during juvenile and adult stages (Hahn, 1989b; Southgate and Partridge, 1998). If the farmed abalone were successfully weaned onto artificial pellets, kelp might not be used throughout the entire culturing period which can save the efforts to collect and clean kelp.

Another benefit of artificial diets is that they produce better growth than macroalgae. Earlier reports showed that growth rates of juveniles fed an artificial feed by Nihon Nosan Kogyo were 65% greater than those fed macroalgae (Hahn, 1989b). Viana et al. (1993) also observed faster growth rates of *H. fulgens* juveniles fed artificial feeds made of local ingredients (fish meal and casein) than those fed macroalgae. In addition, juvenile *H. asinina* fed the artificial diet containing 32.4% crude protein produced greater total body weight and shell length than those fed *Gruciluriopsis heterocludu* in 90-day culturing periods (Emmanuel and Corre, 1996).

The reason behind poor growth has been attributed to the low protein conten of marine marcoalgae, approximately 10-20% crude protein in dry weight, that is virtually unable to meet around 30%-47% protein requirement for some species of abalone (Fleming et al., 1996; Johnston et al., 2005). Marcoalgae, in comparison with artificial formulated diets, does have a relatively low nutritional value (crude protein around 15%) and an unbalanced amino acid profile (Simpson 1994; Britz, 1996b; Erasmus et al., 1997; Rosen et al., 2000). Likewise, wild *Ulva lactuca* has a low protein content, ranging from approximately 3.7-19.9% (Robertson-Anderson, 2003; Naidoo et al., 2006).

Artificial feeds are appropriately formulated so as to supply the necessary protein level and carbohydrate balance ratios required to produce optimum growth in abalone (Middlen and Redding, 1998; Bautista-Teruel et al*.,* 2003). It has been ascertained that protein requirements for *H. midae* are up to 47% (Britz, 1996a), while carbohydrate requirements for most *Haliotis* species range between 43-48% (Sales and Janssens, 2004). All these pellets are all well formulated in order to fulfill the nutritional requirements for several functions--carbohydrates (Nelson et al., 2002) and protein (Fleming et al., 1996; Guzman and Viana, 1998) as a source of lean tissue growth, lipids as a source of energy (Durazo-Beltran et al. 2003) and

regeneration of damaged shells (Nelson et al. 2002). It is not a hyperbole to say that relatively slow growth rates of abalone fed on macroalgae (Britz 1994; Bautista-Teruel and Millamena, 1999; Tan and Mai, 2001) have appositely spurred the development and usage of formulated feeds in the aquaculture industry (Britz, 1996a, b; Sale and Britz, 2001).

New Zealand's aquaculture of *H. iris* is doubtless on the way towards artificial formulated diets. However, published material is scant on the requirements of nutrients on the part of specific *H. iris* when applying artificial diets. This impediment seems likely to have been impeding the development and progress of the country's black-footed abalone aquaculture industry for years. An optimal requirement level of nutrients for abalone has been found as species-specific, and their requirements for protein and lipids have been documented in some studies (Hahn, 1989b; Britz, 1996a; Viera et al., 2005; Sales and Janssens, 2004). This study will allow more scope for further research on the nutrient requirements of *H. iris* in the hope of developing a suitable abalone diet for commercial purposes.

As is well-known, the basic nutrients in a formulated diet are proteins, lipids, carbohydrates, vitamins and minerals. Below is a detailed discussion of each component:

1.5.1 Protein

Protein nutrition is undoubtedly the core of most studies in the field of aquatic animal nutrition (Guillaume et al., 2001). Since the first attempt to use manufactured food for aquaculture, great efforts have been made to define the optimal protein content of diets that aquatic animals need (NRC, 1993). It rapidly became evident that the protein level in aquatic organisms must be higher than that for land mammals or birds. Several complementary routes have been explored in order to explain these particular requirements. Proteins are not only the major constituent of animal's bodies, but also function as enzymes and hormones. Therefore, a continuous supply of proteins with balanced amino acids is needed for maintenance of body metabolism and growth of target animals. Without satisfying protein requirements, adequate growth and normal health of animals cannot be ensured.

On the other hand, if excessive proteins are provided, some of them will be metabolized as energy, but most will not be effectively used. Therefore, great efforts have been made to define requirements, in terms of essential amino acid (EAA), for some fish species and shrimps (Guillaume et al., 2001). Being the most expensive components in the artificial diets (Fleming et al., 1996), the protein supplied in diets should be made as palatable and digestible as possible to save the cost of protein. Thus, protein sources should first be explored and evaluated before incorporation into practical diets. That is why Uki et al. (1985) evaluated abalone growth using diets containing a number of protein sources, including casein, soy bean meal, rye grass concentrate, egg albumin, whole egg and fishmeal. They concluded that casein could be the most suitable protein for inclusion in artificial diets. However, because of its relatively high cost, casein is unlikely to be widely used as a primary protein source in practical diets. The results from Uki et al. (1985) also suggested that commercial fishmeal was not a promising protein simply because the growth rates of abalone fed fishmeal-based diets were approximately half of those fed casein-based diets. However, another study showed abalone growth had considerably improved when they were fed diets containing fresh fish or fishmeal that was not heated during processing (Uki and Watanabe, 1992).

Dietary protein levels also vary between different abalone species. Mai et al. (1995a) estimated that the optimum crude protein level for growth of *H. discus hannai* was between 25% to 37%; Taylor (1997) and Coote et al. (2000) reported that dietary crude protein requirements of *H. kamtschatkana* and *H. laevigata* were 30% and 27%, respectively; *H. midae* exhibited optimum growth rates when crude protein levels were 36% to 47% in the diets (Britz, 1996a).

These optimum dietary protein levels for different abalone species are between 27-44%, which are lower than those for most carnivorous and omnivorous fish, but similar to those of several herbivorous fresh-water fish, such as common carp and Nile tilapia (NRC, 1993). These differences may be due to abalone size, feeding trial periods, the ratios of digestible protein to energy, essential amino acid patterns in the diets, culture surroundings, or statistical analysis methods used in the studies. Differences in leaching rates of nutrients from the diets into water could also have resulted in the differences in the protein requirements, as these studies had revealed**.**

Aside from biologic factors that affect protein requirements of abalone, economical consideration and a culture strategy also need to be examined in practical aquaculture activity.

This seems to be of primary importance to abalone farmers who are keen to grow their abalone to a marketable size. In this light, some alterative protein sources also have been explored in research on formulated diets. The most commonly-used protein sources in abalone feed diets are fish meal, defatted soybean meal, and casein (Guzman and Viana, 1998).

Fish meal is the only protein source that can solely support good growth performance. Others, such as soybean meal and casein need to be fed together with other protein sources in order to support good growth (Fleming et al., 1996). A combination of abalone viscera silage and soybean meal as protein sources in abalone *H. fulgens* diet was found effective in supporting good growth (Guzman and Viana, 1998). Uki et al. (1985), in their search for various protein sources for abalone, identified casein as the most suitable protein. Later, Bautista-Teruel et al., (2003) and Viana et al. (1993) contended fish meal and casein used as protein sources for abalones had achieved similar growth rates and FCR values. The reason for the better growth was mainly attributed to protein quality due to differences in digestibility of each protein source by juvenile abalones. However, because of its relatively high cost, casein is not suitable for practical use in abalone feeds. *Spirulina*, likewise, was shown to have a very good potential as a protein source for abalone (*H. midae*) diets. *H. midae* fed fishmeal and *Spirulina*-based diets exhibited good growth and high efficiency of feed conversion to body weight (Britz et al., 1994; Britz et al., 1996b). Furthermore, plant protein sources such as field peas, faba beans, yellow lupins, defatted soyflour and vetch were tested and used by Vandepeer et al. (1999) for *H. laevigata*. Their results showed that all these legumes were well digested by various abalone species.

However, nutrient research on protein-source levels for *H .iris* is still lacking. That may in part explain the stagnation of the New Zealand abalone industry. The only artificial diet record available was produced by Promak Technology (NZ) Ltd., which used 2-3% of fish meal and 42% of casein to produce the diet. The crude protein level in this diet was up to 53% of the total weight, which produced 90 μm per day growth on shell length in the short run and 30-53 μm per day growth on shell length on the part of *H. iris* with sizes under 20 mm initially under ambient temperature (Fleming et al., 1996). However, there has been no related information about what protein source or at what level it should be applied in a formulated diet for *H. iris.* More detailed discussion on this subject will be carried on in Chapter 4 and Chapter 5 of this study.

1.5.2 Carbohydrate

Carbohydrates are the main components of the macroalgae that fed abalone naturally. They exist in many forms, such as starch, polysaccharides, alginate, agar, amd carrageena in the marco-algae. After digestion by digestive enzymes, the carbohydrates become monosaccharides and are then catabolized for energy. Assays of the hepatopancreatic enzymes of *H. midae* showed that abalone produce their own cellulase, alginate lyase, laminarinase, agarase and carrageenase. A number of studies dealing with the digestive enzymes of abalone have concluded that these animals could synthesize their own cellulase (Elyakova et al., 1981; Boyen et al., 1990; Gomez-Pinchetti and García-Reina, 1993) and alginate lyase (Ostgaard and Larsen, 1993; Ostgaard et al., 1994). All these research supported the notion that carbohydrate is a main energy source for abalone in their natural diets. A result from Tayler (1997) also indicated that carbohydrate in the formulated diets can be used for energy, thus amino acids can use for growth rather than waste on energy metabolism. For abalone, carbohydrates feature top on the list of energy sources rather than lipids or proteins. The carbohydrate requirements for most *Haliotis* species range from 43 to 48% (Sales and Janssens, 2004) in formulated diets.

Carbohydrates in this study came from starch and cellulose. Starch contents in test diets in Chapter 4 and Chapter 5 were fixed at 40%, coupled with varying amounts of cellulose, which could basically fulfill energy requirements of abalone, according to a study by Sales and Janssens (2004). However, the carbohydrate and energy requirement of *H. iris* deserve further research.

1.5.3 Lipids

Both animal and plant ingredients contain a group of substrates, insoluble in water, but soluble in organic solvents such as ether, benzene and chloroform, which are generally referred to as lipids. Lipids can roughly be classified into three groups: glyceryl, ether, and wax (true wax, cholesterol ether, vitamin A or D ethers etc. Guillaume et al., 2001). Phospholipids and carbonsides are the main constituents of compound lipids. The derived

lipids are hydrolytics–end products of simple and compound lipids, with fatty acids as their main constituents.

Judging from a significant amount present in fish bodies and their feeds, neutral fats are by far the most important groups of lipids, but other lipids such as phospholipids also play a significant role in nutrition and physiology. As with carbohydrates and proteins, lipids contain carbon, hydrogen, and oxygen. In contrast, certain phospholipids contain nitrogen as well as phosphorus. Dietary lipids have a huge role to play in providing energy, essential fatty acid (EFA), and fat-soluble nutrients for normal growth of animals. Carnivorous fish have limited ability to use carbohydrates as an energy source, but can efficiently use dietary lipids as high as 10 to 25% (Guillaume et al., 2001). That is why, in a formulated diet for *H. discus hannai*, Mai et al. (1995a) ascertained an optimum lipid requirement of around 3-7%.

As a rule, aquatic animals utilize lipids for energy, for cellular structure, and for maintenance of the integrity of bio-membranes. The fluidity of bio-membranes is regulated partly by the fatty acids of phospholipids that control such processes as cellular transport and activities of membrane-associated enzymes. Most aquatic animals differ from terrestrial ones in that their tissues contain a fairly high amount of n-3 poly-unsaturated fatty acid (n-3 PUFA) such as 20:5n3 and 22:6n3. The degree of fatty acids' unsaturation in tissues increases when environmental temperature is lowered, thereby maintaining membrane fluidity to allow normal cellular function (Guillaume et al., 2001).

The significant role that lipids play in the nutrition of mollusks, especially at larval stages, also has been demonstrated by previous work (Pillsbury, 1985; Whyte et al., 1989, 1990, 1991; Delaunay et al., 1991; Marty et al., 1992; Robinson, 1992). For example, lipids in mollusk larvae have been used as an index for monitoring their physiological and nutritional status, and as a potential for successful metamorphosis (Gallager et al., 1986). However, published material on the quantitative requirement of lipid and its utilization is scant for most mollusk species, including abalones, even if abalones are commercially the most important marine gastropod in aquaculture. Uki et al. (1985) suggested that the optimum lipid level for *H. discus hannai* was about 5% in an artificial diet, but the effect of dietary lipids on its tissue composition was not investigated. One year later, Uki et al. (1986 a, b) went further to explore the requirements of *H. discus hannai* for essential fatty acids

(EFA) and the effect of dietary lipids on the fatty acid profile of abalone tissue. Up to now, lipid requirements and fatty acid requirements for *H. iris* juveniles still remain an unknown quantity.

1.5.4 Minerals and vitamins

Our knowledge of mineral requirement for aquatic animals is limited. Although many studies have been conducted on osmoregulation, heavy metal toxicity and related physiological functions, few of these findings are relevant to nutrition. Unlike other nutrient elements, a significant amount of minerals can be absorbed from the surrounding water, thus making it harder to get proper control of dietary intake of the minerals being studied. Especially in an experimental process of tracing minerals such as iron, zinc, manganese, copper and cobalt, it is very difficult to measure their requirements. On the other hand, this provides a significant benefit for farms except those operating with a closed recirculation water system, because mineral insufficiency will not take place unless an extremely unbalanced mineral diet is provided. Coote et al. (1996) showed that abalone did not require high levels of Ca in their diet, but increasing phosphate supplementation could improve their growth rates. Another interesting element is zinc, which was found to improve both growth rates and the body's alkaline phosphatase activity (Tan and Mai, 2001).

Few studies have been conducted on vitamin requirements for abalone. The effect of dietary vitamin C on survival, growth and tissue concentration of ascorbic acid was investigated by Mai (1998), who claimed that dietary ascorbic acid (AsA) had clear effect on the carcass AsA concentration, but had no significant effect on growth and survival. Furthermore, Tan and Mai (2001) suggested that vitamin K might contribute to mineralization of shells in abalone. In the present study, vitamin and mineral premix was each fixed at 1.5% w/w in experimental diets that were used in Chapter 4 and Chapter 5.

1.6 Environmental Parameter and Nutrients

In abalone aquaculture, there are some biotic and abiotic factors that closely relate with abalone farm management. High dissolved oxygen, stable pH, fixed salinity, and suitable

water temperature have been identified as critical factors for managing abalone farms (NRC, 1993). In New Zealand, a recirculation style has been followed in most abalone farms. The water in this system is reused for many times before it is discharged, and consequently all these parameters will not be sufficient enough for the abalone's daily requirements. In this context, it is essential for farmers to re-oxygenize the water, adjust pH, control increased salinity resulting from water evaporation, and maintaining the temperature of the system balance against the effects of the surrounding environment.

1.6.1 Dissolved oxygen (DO)

Oxygen consumption by intensively cultured abalone greatly affects oxygen concentration in rearing water. Abalone's oxygen consumption has been shown to be affected by water temperature, body size and time of day (Hahn, 1989c). Tayler (1997) reported that during resting times, abalone *H. kamatschatkana* consumed 20.7 µl O_2 g^{-1} h^{-1} under 12 °C ambient temperature. When the abalone were cultured at high density or in recycled water, extra aeration or pure oxygen input was needed to maintain constant DO values to fulfil abalone requirements. Yang and Ting (1989) indicated that dissolved oxygen (DO) should be maintained higher than 5 mg /L for the optimal growth of *H. diversicolor supertexta*. They also reported that the oxygen consumption of this species cultured at 30-32 °C was 1.3 times greater than that cultured at same salinity and 25 °C. Fallu (1991) argued that abalone require greater DO than 3–4 mg/L of water, and that low DO treatments for 24 hour would upset the abalone's immune system, as proposed by Cheng et al. (2004). Low DO treatments at low levels of 3.57 and 2.05 mg/L caused depression in the immune system of *H. diversicolor supertexta*, and increased their susceptibility to pathogenic bacteria *Vibrio parahaemolyticus* infection (Cheng et al., 2004).

1.6.2 pH value

The pH values in recycled water will decrease over time in ongoing recirculating systems. A drop in pH is mainly caused by the generation of nitrogen waste and accumulated CO_2 in the water. The pH drop leads to shell erosion of juvenile abalone. Erosion is first visible at the shell apex and then the shell slowly erodes until exposing the nacreous layers. As a result, the shell turns out to be very fragile and breakable (Hahn, 1989c, Sakai, 1980).

The problem can be fixed by adding lime or NaOH into the system or introducing new water into the system. Maintaining stable pH at 8.0-8.3 is a main challenge for abalone farmers.

1.6.3 Salinity

As all abalone are marine species, fresh seawater is doubtless the most common media for abalone aquaculture. In New Zealand, artificial seawater is sometimes used in a full recirculation system, however natural seawater still preponderates in the selection of culture media. Although the salinity of abalone cultures should be maintained exactly as seawater salinity, many species can countenance little change to salinity in a gradual manner. Japanese abalone *H. discus hannai* larvae have shown a very narrow range of optimal salinity, which is between 31.8 and 33.4‰. However, *H. sieboldii* larvae have a much wider range of 30.8-36.3‰ (Hahn, 1989c).

As with other physical factors, salinity also is influenced by temperature. High temperature will cause high evaporation speeds and lead changes in the salinity of re-circulating systems. Temperature and salinity are the primary physical factors affecting the life of mollusks. It is reported that salinity in the range of 30–35‰ and temperature in the range of 24–30 °C are the optimal levels for the growth of *Haliotis diversicolor supertexta* (Chen, 1984). *H. diversicolor supertexta* reared at 20, 25 or 30 °C could correspondently survive a salinity range of 4.5–33.1, 7.7–33.7 or 13.5–36.5‰ when water temperature was decreased or increased gradually (Chen and Chen, 1999). Their survival salinity ranges moved up and down with an increase or decrease in temperature (Chen and Chen, 1999, 2000). Environmental physical-chemical changes, such as temperature and salinity and environmental pollutants have been reported to affect the circulating haemocytes and phagocytosis in several species of mollusks, including red abalone *H. rufescens* and black abalone *H. cracherodii* (Fisher et al., 1987; Pipes and Coles, 1995; Martello et al., 2000). Maintaining a stable salinity also is one of the most important tasks on abalone farm with recirculation systems in New Zealand.

1.6.4 Temperature

Temperature is one of the key factors governing physiological processes of all organisms. It has effects even on other physical factors such as DO, pH and salinity. It works at the cellular level by boosting or diminishing the catalytic activity of metabolic and digestive enzymes (Hochachka and Somero, 1984). Temperature maintains a direct relationship with growth rate and other whole-body functions involved in energy metabolism (respiration, food consumption, excretion, etc.) (Prosser, 1991), somatic growth (Leighton et al., 1981; Britz et al., 1997; Hirst and Bunker, 2003) and rate of maturation exhibited (Morse, 1981; Browne and Wanigasekera, 2000; Halsband-Lenk et al., 2002). It is generally believed that temperature is a good indicator of seasonality in marine environments (Hairston and Kearns, 1995).

In New Zealand, surface sea temperature (SST) is hugely different around different areas, which may be associated with sea currents and land distributions. In summer, surface sea temperature ranges from a high of around 20 °C in the north to a low of just below 13 °C in the south. The coldest surface water in summer, about 9.5 °C, occur off North Canterbury, and water temperature remains around 10 °C southwards as far as south Otago. In winter, the warmest water is just over 16 °C, looking like a series of narrow vertical streaks west of the Northland Peninsula (Mullan, 1998).

In this study, fresh seawater was collected from outside Otago Harbour (Chapter 4) and inside Otago Harbour (Chapter 5). Because of high exposure to winds, the water temperature of Otago Harbor is sometimes lower than the average SST of Otago area, as much as 7.5 °C in historical records. In Chapter 4, seawater was preheated and fixed at 18 °C which simulated Otago temperature in summer. In Chapter 5, ambient temperature and 5 °C higher temperature were applied to the tests, which simulated New Zealand's seasonal difference of 5 °C as Mullan (1998) described.

The only investigation on the effect of temperature on *H. iris* was published by Searle in 2004, which clearly stated the optimal temperatures for growth are 21-22, 20-21 and 17-18 °C for the 10, 30 and 60 mm size classes of juvenile black-footed abalone, respectively. In comparison with the sea temperature, North Island's SST seems more suitable for abalone aquaculture. Searle (2004) reported that *H. iris* juveniles produced 50% mortality when the culturing temperature rising up to 28.8, 27.7 and 27.8 °C for the 10, 30 and 60 mm size-

classes of abalone. Most areas in New Zealand are unlikely to be highly affected by large fluctuations of water temperature as tropical areas can be, but some areas in the upper North Island where water sourced from shallow bays will be affected by solar heating, especially during mid-summer.

Temperature also has a role to play in abalone growth. Brize et al. (1997) investigated the growth of *H. midae* by increasing the temperature from 12 to 20°C and observed some improvements in growth rates, and feed consumption increased. The protein-efficiency ratio (PER) improved, and FCR did not differ significantly. However, when the test temperature was raised to 20 and 24°C, the result was significantly different, with growth rates and feed consumption declining sharply, and PER and FCR deteriorating.

There is no literature focusing on thermal regulation of *H. iris* even though there is a huge temperature difference between north and south, summer and winter sea temperature. In New Zealand, the differentials in sea water temperature may be as wide as 5-10 °C in different regions and in different seasons. In the present study, two temperature ranges simulating North Island's water temperature--ambient Otago water and 5 degrees higher than that--were applied to different levels of diets. That was based on a hypothesis that under diverse temperature environments *H*. iris could have different protein requirements.

1.7 Aims of Study

Protein nutrition is undoubtedly essential to aquatic organisms. Since the first attempt to use manufactured food in fish culture, great efforts have been made to define the optimal protein content of diets. However, many abalone farms have to use large quantities of a variety of macroalgae (Hahn, 1989b) to fulfill nutritional requirements (Britz, 1996a, b; Viera et al., 2005). However, macroalgae unfortunately have a relatively low protein content (around 15%) and an unbalanced amino acid profile (Troell et al., 2004) of limited nutritional value (Hahn 1989b), which are becoming limited factors for farm operation (Hecht and Britz, 1990; Viera et al,. 2005; Johnston et al., 2005). These problems have prompted researchers to investigate the production and use of artificially formulated diets.

In this context, the aim of this study is to look into the protein requirements of New Zealand's black-footed abalones (*H. iris*). This will provide a way for designing a better, inexpensive, artificial diet recipe to improve both survival and growth rates of abalones to meet the growing demand of the market.

In the present study, different protein sources that are all very easy to obtain in New Zealand were first tested in growing-out experiments to certify that they were the preferable protein sources for *H. iris*. Then the most preferable protein was picked for protein level experiments to look at the effect of water temperature and protein requirements.

This study also attempted to evaluate the role nutrients play in the amino acid composition of abalone's shell and soft-body. By using different protein levels and water temperatures, changes in amino acid composition of the shell and body were analyzed. Meanwhile, this research examined amino acid requirements of New Zealand's black-footed abalones (*H. iris*) in both larval and juvenile stages by using different strains of microalgae (with diverse amino acid contents) that were isolated from New Zealand coasts.

The framework of this thesis, chapter-by-chapter is listed below:

Chapter 1 provides a general introduction to the biology, ecology, fisheries and aquaculture practices of abalones worldwide and in New Zealand.

Chapter 2 looks at the role young and mature single strain microalgal biofilms play in *H. iris* larval settlement, as well as the role nutrients, particularly amino acids play in early life of abalone larvae.

Chapter 3 looks at the role of mixing dual-strain microalgal biofilms in *H. iris* larval settlement, as well as the role of nutrients, particularly amino acid in early life of abalone larvae.

Chapter 4 is designed to explore suitable protein sources for *H iris* in formulated diets with a view to finding the best sole protein sources for *H. iris* aquaculture. Nine local protein sources were tested as sole protein source, including some unbalanced amino acids plant proteins. This chapter also seeks to answer whether diets could lead to amino acid deficiencies. Shell protein and soft body tissue amino acid compositions also were monitored to compare the effect of different dietary proteins. Protein sources with best preferences were then used in the next chapter.

Chapter 5 aims to tease out the relationship between different temperature regimes and abalone protein requirements. Different indices in soft-body tissue, shell, histological feature, and proximate analysis data were compared in different treatments. Shell protein and soft body tissue amino acid compositions also were monitored to compare the effect of temperature or protein levels. Second-order polynomial curves were used to estimate protein requirements under different temperatures.

Chapter 6 is designed to explore the morphological variations in shells that are due to different dietary protein sources and different levels of protein in their diets. The effect of temperature was also tested.

Chapter 7 provides a general thesis discussion and conclusions with recommendations for industry development according to the results of the present study. Future possible research avenues also are put forth.

Chapter 2 Initial attachment, metamorphosis, settlement, and survival of Black-footed Abalone (*Haliotis iris*) on microalgal biofilms containing different amino acid compositions

2.1 Abstract

Microalgal biofilms with different amino acid profiles were investigated for their ability to induce abalone (*Haliotis iris*) attachment, metamorphosis, settlement, and survival. Twenty microalgal strains, isolated from rocky shores and sandy estuaries, were grown in the laboratory to produce young and matured monospecific biofilms. Abalone larvae were exposed to the different biofilm treatments and controls (no biofilms) for 7 (attachment and metamorphosis) and 14 (settlement and survival) days. The larvae performed significantly better in biofilms compared to controls across microalgal strains, but attachment, metamorphosis, settlement, and survival were generally less than about 50, 35, 25, and 25%, respectively. Some microalgal strains belonging to the same species but collected from different sites had different effects on the larvae, likely due to variations in biochemical composition and activity among strains. While no clear relationship was found between the microalgal amino acid profiles and larval processes, percent biofilm cover and total amino acids were positively correlated with some of these processes. In addition, abalone performed significantly better when exposed to mature compared to young biofilms. These results may be due to the greater amounts of microalgal cells and their extra-cellular polymers within older biofilms, which may cue larvae toward more nutritionally favorable environments.

2.2 Introduction

Commercial cultivation of abalone usually is conducted within tanks previously inoculated with microalgal films as grazing surfaces. These films (biofilms) often contain one or more microalgal species, which may induce metamorphosis and settlement, and may improve survival and growth of young abalone (Kawamura et al., 1998; Gordon et al., 2004; 2006). Thus, the chemical and nutritional composition of the biofilm is of crucial importance for abalone hatchery production. The biofilms themselves are highly complex structures or communities of organisms encapsulated within a self-developing polymeric matrix, and adherent to a living or inert surface (O'Toole et al., 2000). A variety of microorganisms, such

as bacteria, protozoa, fungi and microalgae may be contained within the biofilm (Faimali et al., 2004). Each group of microorganisms may perform its own specialized metabolic function, which in turn may be modulated by neighboring organisms and their extracellular products (Harder et al., 2002a, b; Karatan and Watnick, 2009).

In the marine environment, such multi-species biofilms cover nearly every living and inert surface, and play an important role in the lives of marine invertebrates and the food webs in which they are a part (Pawlik, 1992). Two important biological processes, which are mediated by biofilms, are larval settlement and post-larval grazing. Metamorphosis – a developmental change that occurs just prior to settlement in many marine invertebrates such as abalone – also is triggered by chemicals within adjacent biofilms (Mihm et al., 1981; Wahl, 1989). Thus, larval metamorphosis and settlement are known to be influenced by chemical signals produced by biofilms in both wild and hatchery conditions (Wieczorek and Todd, 1997, 1998; Roberts et al., 2007). Laboratory studies of monospecific biofilms and isolated chemicals, such as gamma-aminobutyric acid (GABA) and potassium chloride, have highlighted the importance of a range of chemicals for settlement, which may be produced by different biofilms (Morse and Morse, 1984a; Morse et al., 1984; Bryan and Qian, 1997; Roberts, 2001a; Lam et al., 2005). Recent studies have demonstrated that mono-specific bacteria or diatom biofilms can induce larval metamorphosis and settlement in bryozoans (Dahms et al., 2004), polychaetes (Unabia and Hadfield, 1999), mussels (Satuito et al., 1995; 1997), oysters (Fitt et al., 2005), barnacles (Maki et al., 1990), and sea urchins (Huggett et al., 2006). For example, Huggett et al. (2006) isolated 250 bacterial strains from coralline algae, and found that some bacteria, such as *Pseudoalteromonas luteoviolacea*, had a strong ability to induce settlement of sea urchins (*Heliocidaris erythrogramma*). Using abalone (*H. iris*) larvae in 4-day experimental trials, Roberts et al. (2007) also found that 16 monospecific diatom biofilms generated over 80% attachment and one monospecific biofilm produced over 50% metamorphosis.

Research on the inductive properties of certain biofilms has led to use of cultured microalagal and bacterial biofilms within hatchery environments for abalone production (Kawamura et al., 1998; Daume et al., 1999; Roberts, 2001a; Gallardo and Buen, 2003). Such biofilms often are dominated by one or more microalgal species which produce large amounts of extra-cellular polymers (ECP). These ECP products contain chemicals that also have been shown to induce settlement in abalone (Daume et al., 2000; Chen, 2007). For example, Chen

(2007) found that soluble extracellular polymers improved the survival rate of small abalone (*H. diversicolor*) post-larvae.

Once settled, abalone post-larvae immediately begin to graze the surrounding biofilm. This initial grazing activity is thought to be critical for their survival and growth (Takami and Kawamura, 2003). At this stage, the dominant microalgal species in the biofilm may determine the success or failure of the hatchery production. Few microalgal species have been shown to be consistently suitable for abalone hatchery production (Roberts 2001b), and most that have been tested have been found to be unsuitable for abalone hatchery production (Daume et al., 1999; Roberts, 2001a). This is probably due to the fact that an appropriate food for abalone post-larve needs to have the appropriate shape, size, and nutritional value for the grazer (Kawamura et al., 1998). Thus, improvements in the survival and growth of newly settled abalone require a carefully designed biofilm containing palatable, digestable, and nutritious microalgal species. Several studies have investigated survival and growth of abalone post-larvae with different microalgal species with various results (Kawamura et al., 1998; Roberts, 2001b; Chen, 2007). For *H. iris* larvae, Kawamura et al. (1998) suggested that differences in larval survival are related to food digestion efficiency which, in turn, is influenced by diatom characteristics, such as morphology, attachment and frustule strength. Conversely, Roberts et al. (2007) found that diatom physical characteristics were less important for *H. iris* attachement and metamorphosis compared to the amount and age of the biofilm. However, studies based on the nutrition level of microalgae are lacking. It is possible that the reason for the poor performance with previously-tested microalgae is that most hatchery productions use commonly cultured microalgae, which are not necessarily the most nutritious for abalone post-larvae.

In addition, the age of the culture appears to be a determining factor in the success of larval rearing (Wieczorek et al., 1996; Faimali et al., 2004). Faimali et al. (2004) investigated changes in biofilms over time, and found that as biofilms matured and increased their diatom populations, their ability to induce larval settlement increased. Wieczorek et al. (1996) found that various species of invertebrate larvae performed better when grown in a well-aged biofilm compared to a newly formed biofilm. However, the actual reasons why matured biofilms improve larval conditions are still unclear. One potential factor is variations in the amino acid composition among microalgal species, and their changes over time, which may directly relate to the culturing efficiency of abalone post-larvae. Thus, the aim of this study is

to determine the effect of amino acid composition (as a proxy for nutrition) in biofilms of different microlagal strains and ages (young and matured) on abalone (*H. iris*) attachment, metamorphosis, settlement, and survival.

2.3 Materials and Methods

2.3.1 Microalgal collections, isolations, and cultures

Benthic microalgal samples were gathered and isolated from four rocky and beach coasts around Auckland Harbour (North Island, New Zealand) during low tide periods between June and September 2006. The sampled sites included rocky at Devonport [36°49'45"S, 174° 48'23"E] and Bucklands Beach [36°51'36"S, 174°53'59"E], and sandy at Point Chevalier [36°51'4"S, 174°42'11"E] and Eastern Beach [36°52'3"S, 174°54'41"E] seafloor environments. Each microalgal sample was cultured in a 9 cm diameter polyethylene Petri dish with 25 mL F_2 media (Guillard and Ryther, 1962; Guillard, 1975). Single-strain cultures were obtained by purifying the samples under a 1200 lux light source with 12 light/12 dark cycles and a constant temperature environment (15±1 °C). The isolated microalgal species were identified based on their morphological and cell division characteristics under a light and electron microscope (Austrid, 1968; Humm and Wicks, 1980; Round et al., 1990). Twenty identified microalgal strains, representing 13 different species, were classified according to distinct morphological characteristics (Table 2.1).

These microalgal strains were cultured in 20 mL F_2 medium for 14 days. After this growth period, the enriched seawater was decanted from each plate, and the benthic microalgae (attached to the bottom of the plate) were scraped off and re-suspended in fresh F_2 media. From these solutions, 2 mL from each microalgal strain were introduced into one of 3 Petri plates with F_2 media to make up 20 mL solutions, and left for 7 days to produce microalgal biofilms. Previous experiments had shown that these microalgal strains require 7 days to form a biofilm.

2.3.2 Larval settlement experiments

H. iris larvae of about 7 to 8 days old were obtained from Oceanz Blue Ltd., at Bream Bay Aquaculture Park, northern New Zealand, and transported to the Auckland University of Technology lab in filtered seawater (Milipore™, 0.45 μm). Immediately upon arrival, 25-35 larvae in 5 mL filtered seawater were added to the experimental Petri dishes, containing each of 20 single-strain microalgae cultured (Table 2.1) for only 4 hours (young biofilm) and 20 single-strain microalgae cultured for 7 days (matured biofilm). Three controls (no microalgae added) were used for each of the 2 single-strain sets of experiments. Three replicates per treatment and control were used. Larval attachment (initial attachment to the substrate, but cilia still visible) and metamorphosis (appearance of cephalic tentacles and complete disappearance of cilia) were recorded after 7 days, and settlement (sustained attachment to the substrate, peristomal shell starts to form and grow on the right side of the animal) and survival were recorded after 14 days.

2.3.3 Amino acid analyses

The amino acid composition of each microalgal strain cultures (young and matured biofilms) was determined by scraping microalgae from the bottom of three culture Petri dishes for each strain. All samples were placed inside individual 25 mL plastic test tubes with lids, and centrifuged at 2000g for 10 minutes. The amino acid analysis was conducted in accordance with Paramás et al. (2006). The microalgal precipitates were mixed with 5 mL of water in 25 mL screw top glass tubes. To each sample, a 5 mL 13M concentrate of hydrochloric acid with 0.1% phenol solution was added, and heated in a Velp digester at 110 °C for 12 hrs under nitrogen gas. After cooling, the solution was adjusted to a pH of 6.5-7.5 by adding 1M NaOH solution. The final volume was recorded and stored in a -20°C freezer until further analysis.

In order for the amino acids to fluoresce in the HPLC, the samples were reacted with o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (MCE) at room temperature, which provided isoindolic derivatives. The OPA reagent was prepared by dissolving 500 mg of OPA (Merck) in 22.5 mL of ethanol. A total volume of 25 mL OPA solution was obtained by adding 0.4M borate buffer (pH 10) and 400 μL of 2-mercaptoethanol (Sigma). A 50 μL

volume of each sample was mixed with 450 μL sodium phosphate buffer (100 mM, pH 7.3; BDH), 100 μL OPA reagent, and 50 μL of an internal standard (3, 5-dibromotyrosine) in a new 1.5 mL vial. The final internal standard concentration in the sample solution was $1x10^{-5}$ M. These mixtures were prepared just prior to analyzing with HPLC.

A mixed L-amino acids standard was prepared each day to calibrate the HPLC before running the samples. This standard was prepared in sodium phosphate buffer, and contained leucine (Leu) 1.844 g/L, isoleucine (Ile) 0.879 g/L, methionine (Met) 0.577 g/L, taurine (Tau) 0.189 g/L, tyrosine (Tyr) 0.125 g/L, glycine (Gly) 0.236 g/L, valine (Val) 0.925 g/L and Proline (Pro) from BDH; aspartic acid (Asp) 0.059 g/L, alanine (Ala) 0.007 g/L, and phenylalanine (Phe) 0.354 g/L from Merck; and γ-aminobutyric acid (Gaba) 1.650 g/L, ornithine (Orn) 0.930 g/L, a-aminoadipic acid, histidine (His) 1.459 g/L, lysine (Lys) 0.361 g/L, glutamic acid (Glu) 0.272 g/L, threonine (Thr) 0.781 g/L, arginine (Arg) 0.381 g/L, serine (Ser) 0.395 g/L, and 3,5-dibromotyrosine (I.S.) 1.785 g/L from Sigma.

A high-pressure liquid chromatography (HPLC) system was used to analyze the samples. The HPLC was attached to a Shimadzu LC-10AD automatic sampler fitted with a DGU-2A helium de-gas system. The solid phase consisted of a NOVA-PAK® C_{18} column (4 μm 3.9 × 150 mm, Waters). The mobile phase was adapted from Paramás et al. (2006) to provide a gradient elution. The first mobile phase was 80:19:1 ratio of sodium phosphate buffer (10mM, pH 7.3) /methanol/ tetrahydrofurane (solution A). The second mobile phase contained a 20:80 sodium phosphate buffer (10mM, pH 7.3)/methanol (solution B). Solution A was added at 100% at 0.1 mL/min for the first 3 min, after which time the flow rate was increased to 1.5 mL/min. At 14.5 min, the gradual addition of solution B was started. At 19.5 min, the ratio of solution A to B was 85:15. At 24.5 and 45 min, the ratios were 70:30 and 30:70, solution A to B, respectively. Fluorimetric detection of amino acids was obtained at excitation and emission wavelengths of 340 and 426 nm, respectively.

Because HPLC does not detect proline, visible spectroscopy was employed to the same sample extracts prior to pre-treatment with OPA to detect this amino acid, according to Amerine and Ough (1980). A 0.1 mL subsample of the previously hydrolyzed sample was transferred to a 15 mL screw-top test tube. To this subsample, 5 mL of pure water, 0.25 mL formic acid, and 1 mL methyl cellosolve (2- methoxyethenol) ninhydrine solution (3 g of

ninhydrine dissolved into 100 mL 2- methoxyethenol) were added and boiled with the cap for exactly 15 min in a water bath (Grant, SBB14). After cooling to room temperature, 1 mL isopropanol-water mixture (1:1 by volume) was added. These solutions were transferred into cuvettes to measure absorbance with a spectrophotometer (Ultrospec 21000pro UV/Visible) at a 517 nm wavelength. The results were calculated on the basis of the curve formed by the standard solution.

A cell count for each microalgal culture (young and mature) was used to standardize individual amino acid weights per cell (TAA). Distinction between essential and non-essential amino acids was made following Allen and Kilgore (1975). Threonine (Thr), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Phenylalanine (Phe), Lysine (Lys), Histidine (His), and Arginine (Arg) are deemed essential amino acids (EAA), while Glutamic acid (Glx), Aspartic acid (Asx), Alanine (Ala), Taurine (Tau), Glycine (Gly), Serine (Ser), and Proline (Plo) are non-essential amino acids (NEAA). The total amino acid weight was calculated by adding the weight of all essential and all non-essential amino acids. EAA: NEAA ratios were calculated by dividing the total EAA weight by the total NEAA weight. In addition, the percent cover of biofilm within each Petri plate was obtained by multiplying the mean cell count per cm^2 by the mean cell size, and assuming cells were elliptical (Roberts et al., 2007).

2.3.4 Statistical analyses

A Kruskal-Wallis test was performed on attachment, metamorphosis, survival and settlement data (microalgal strain as fix factor), followed by Dunn's multiple comparisons. Comparison of larval parameters between young and mature bioflim treatments also were performed with Kruskal-Wallis tests. Pearson correlations were performed between percent attachment, metamorphosis, survival and settlement with percent biofilm cover, TAA, EAA, NEAA, and EAA/NEAA ratios. All analyses were performed using MINITAB version 14 software package.

2.4 Results

2.4.1 Larval attachment after 7 days

Abalone larval attachment was highly variable among biofilm treatments, but generally higher than controls (Fig. 2.1a, Table 2.2), indicating the importance of microalgae in the attachment process. For the young biofilm, larval attachment was relatively similar among most microalgal strains, with no statistical differences among strains (Table 2.2). However, *Cylindrotheca* cf. *closterium* (Cyc3) resulted in higher larval attachment (36.06±5.73%) than any other microalgal strain, followed by *Entomoneis* cf. *alata* (Ena1) (25.10±16.52%). In addition, clear differences were observed between some strains within a single species (Fig. 2.1a, Table 2.2). For example, *Cylindrotheca* cf. *closterium* (Cyc2) resulted in poorer larval attachment compared to *Cylindrotheca* cf. *closterium* (Cyc3), which was isolated from a different site. Another example was a difference in the cynobacterium *Oscillatoria* cf. *erythrata* (Ose1 and Ose2), which resulted in high larval attachement with Ose2, but extremely poor attachment with Ose1 (less than 1.5 %, which was even lower than controls).

Larval attachment within matured biofilm treatments was significantly higher than with young biofilm treatments (Fig. 2.1a, Table 2.2-3). However, for all microalgal strains, attachment within matured biofilms was highly variable and significantly higher than controls. *Schizothrix* cf. *calcicola* (Scc1) resulted in the highest larval attachment of 81.74±9.40%, followed by *Anacystis* cf. *aeruginosa* (Ana) with 57.55±1.28%. Clear differences in larval attachment were observed between *Schizothrix* cf. *calcicola* (Scc1 and Scc2), both of which were collected from different sites. Results from Dunn's multiple comparison tests indicate that all microalgal strains were not significantly different from one another, except for the control, which differed from all microalgal strains, and Scc1, which did not differ from Ana only.

2.4.2 Larval metamorphosis after 7 days

The percent larval metamorphosis after 7 days was generally low or zero for young and matured biofilm treatments and controls (Fig. 2.1b, Table 2.2). Out of three microalgal strains that induced metamorphosis, *Cylindrotheca* cf. *closterium* (Cyc2) resulted in the

highest value (13.07±8.35%), which was significantly different from the other two strains (Dunn's multiple comparisons). In general, matured biofilms produced significantly higher percent metamorphosis than young biofilms (Table 2.3). Out of nine microalgal matured biofilms that induced metamorphosis, *Cylindrotheca* cf. *acicularis* (Cya1) and *Navicula* cf. *minuscule* (Nam) performed well, with values of 32.37±19.33, and 31.62±25.91%, respectively. Dunn's multiple comparison tests resulted in significantly different percent larval metamorphosis for Cya1 and Ena2 that was different from the other microalgal strains. For both young and matured biofilm treatments, percent larval metamorphosis in the controls was zero.

2.4.3 Larval settlement after 14 days

Percent larval settlement after 14 days was extremely low (< 10%) in young biofilm treatments, and slighly higher in matured biofilm treatments (< 20%) (Fig. 2.2a, Table 2.3-4). None of the controls had any larval settlement. For the young biofilm treatments, about half of the microalgal strains promoted settlement, and only two strains achieved larval settlement values greater than 5%. *Navicula* cf. *atomoides* (Naa) and *Oscillatoria* cf. *erythrata* (Ose2), respectively, reached 9.23±5.83 and 5.40±3.21% larval settlement. However, a significantly better settlement was found in matured biofilms (Table 2.4). More than 11 microalgal strains promoted larval settlement over 5%. The highest settlement was observed with *Anacystis* cf. *aeruginosa* (Ana), which reached 24.02±13.59%.

2.4.4 Larval survival after 14 days

Larval survival after the 14-day experiments was relatively low (< 25%) in the young and matured biofilms, and none of the larvae survived in any of the controls (Fig. 2.2b, Table 2.3-4). Within young biofilm treatments, *Navicula* cf. *atomoides* (Naa) had the highest larval survival with 15.17±6.59%. *Anacystis* cf. *aeruginosa* (Ana) and *Cylindrotheca* cf. *closterium* (Cyc1) had the highest larval survival within matured biofilm treatments, with 24.02±13.59 and 15.25±1.87%, respectively. The percent survival within matured biofilms was significantly higher than within young biofilms (Table 2.4).

2.4.5 Amino acid compositions

The percent cover of microalgal biofilms increased from 7 to 14 days and from 14 to 21 days for all microalgal species (Fig. 2.3). Since the larval experiments were started after the microalgae were cultured for 7 days, the 7-, 14-, and 21-day microalgal culturing times correspond to 0, 7, and 14 days of larval experiments. Significant differences were observed in biofilm cover among the different microalgal strains. The greatest percent cover was found within *Cylindrotheca* cf. *closterium* (Cyc), *Cylindrotheca* cf. *acicularis* (Cya), *Biddulphia* cf. *aurita* (Bia), and *Entomoneis* cf. *alata* (Ena) cultures (Fig. 2.3). In fact, some of these cultures reached over 100% cover (i.e., cells grew on top of one another). For example, *Cylindrotheca* cf. *acicularis* had 134 and 136% cover for Cya1 and Cya2, respectively; *Cylindrotheca* cf. *closterium* had 152, 163, and 163% cover for Cyc1, Cyc2, and Cyc3, respectively; and *Biddulphia* cf. *aurita* had 125% cover.

The amino acid profiles for all microalgal strains grown for either 7 or 14 days had considerable variability in the amount of individual amino acids, TAA, EAA, and NEAA values, EAA/NEAA ratios, and percent biofilm cover (Tables 2.5-6). In general, the amount of GABA was low for all 7- and 14-day old microalgal strains. In addition, different strains within the same microalgal species, but collected from different sites, differed from one another. For example, *Oscillatoria* cf. *erythrata* amino acid amounts differed greatly between Ose1 and Ose2 for the amino acids Asx, Glx, Thr, and Ala+Tau for the 7- and 14-day biofilms (Tables 2.5-6).

Changes in amino acid compositions between 7- and 14-day microalgal cultures revealed significant differences between young and matured biofilms for 13 amino acids, TAA, EAA, NEAA EAA/NEAA, and percent biofilm cover (Table 2.7). Among these amino acids, their proportions increased for Ser, Gly, Thr, Arg, Val, and Orn, while the proportions decreased for Glx, His, Ala+Tau, Tyr, Phe, and Lys. In addition, the proportions of TAA, NEAA, and percent biofilm cover increased, and the proportions of EAA and EAA/NEAA decresed from the 7- to 14-day cultures.

Peason's correlations between TAA, EAA, NEAA EAA/NEAA and percent biofilm cover, and larval attachment, metamorphosis, settlement and survival resulted in significant

positive correlations for TAA with larval attachment and metamorphosis (Table 2.8). Percent biofilm cover also was significantly correlated with larval attachment, metamorphosis, and survival (Table 2.8). All other relationships were either non-significant or the analyses were not possible due to insufficient data.

2.5 Discussion

2.5.1 Effect of microalgal strain on abalone larvae

Abalone larvae performed considerably better in the presence of microalgal biofilms compared to controls (no biofilm), and some differences were apparent in larval attachment, metamorphosis, and survival among microalgal strains. However, none of the 20 tested microalgal strains resulted in excellent larval performance. In fact, less than about 50, 35, 25, and 25 percent attachment, metamorphosis, settlement, and survival, respectively, were observed across microalgal strains. Based on these results, it appears that these monospecific microalgal biofilms, by themselves, are not conducive to abalone larval rearing. However, some important trends are apparent from these larval experiments.

Percent microalgal cover was correlated with larval attachment, metamorphosis, and survival. In other words, those microalgae which grew better (e.g., *Cylindrotheca* cf. *closterium*, *Cylindrotheca* cf. *closterium*, *Biddulphia* cf. *aurita*, and *Entomoneis* cf. *alata*) generally resulted in higher percent larval attachment, metamorphosis, and survival. These results suggest that higher percent attachment and metamorphosis is achieved in the presence of more abundant extra-cellular polymers (ECPs), which are produced by more developed microalgal biofilms. ECPs from diatoms have been shown to induce attachment and metamorphosis in sea urchins (Lam et al., 2005). In addition, Roberts et al. (2007) found that percent *H. iris* metamorphosis (and to some extent larval attachment) increased when exposed to older microalgal biofilms, which would have produced more ECPs. The positive correlation between percent survival and percent biofilm cover is likely to be due to the more abundant food supply available for post-larvae to graze upon. Indeed, other abalone species, such as *H. asinina and H. discus hannai*, have been shown to grow more slowly when food supplies are low and *vice versa* (Miyamoto et al., 1982; Tahil and Juinio-Menez, 1999). The fact that percent settlement did not correlate with percent biofilm cover may be related to other factors, such as specific chemical cues and/or nutrient requirements which were not

present in the experimental treatments. For example, abalone settlement is thought to be induced by chemicals emanating from coralline algae, although the particular chemicals have not been clearly identified (Morse and Morse, 1984a; Morse et al., 1984; Daume et al., 2004).

Results also showed that different microalgal strains belonging to the same species, but collected from different sites, clearly can differ in their ability to induce larval attachment, metamorphosis, settlement, and survival. These differences are likely to be due to variations in biochemical composition and activity among strains. Such biochemical variations are common among microalgae found in different environmental conditions, and often arise quickly through mutations from one generation to another (Ewart and Pruder, 2009; Rasoul-Amini et al., 2009). Roberts et al. (2007) also found that a microalgal strain (*Cocconeis scutellum*) collected from different locations resulted in significant differences in diatom density and ability to induce larval attachment in *H. iris*. Such unique biochemical characteristics may be reflected in the great variability of inductive abilities found in different microalgal strains in the present study, and highlight the importance of chemical cues rather than morphology in the induction of larval attachment, metamorphosis, settlement, and survival.

No clear relationship was found between the amino acid profiles of different microalgal strains and their ability to induce larval attachment, metamorphosis, settlement, and survival. These results suggest that amino acids do not significantly affect early larval processes, or the amino acids, which are involved in larval attachment, metamorphosis, settlement, and survival, were not present in sufficient quantities in the tested microalgal strains. For example, GABA was present in extremely low concentrations in all the treatments. GABA (~1 µM concentration in the water) has been shown to induce *H. iris* settlement under laboratory conditions (Roberts, 2001b). Other studies also have shown that GABA exuded by coralline algae after damage to algal surfaces stimulates settlement in *H. rufescense* (Morse and Morse, 1984a; Morse et al., 1984; Morse, 1992) and *H. iris* (Roberts, 2001a). However, Kasper and Mountfort (1995) suggested that GABA naturally released from coralline algae after adult grazing could not induce larval settlement, since GABA degrades as soon as it is released into the water. Further studies would need to be conducted to elucidate if GABA within microalgae can induce abalone larval processes.

The fact that TAA correlated well with percent attachment and metamorphosis suggests that the overall nutritional value of microalgae is involved in these larval processes. Since higher amounts of TAA reflect more protein, it is possible that abalone larvae respond to this stimulus by attaching and metamorphosing in environments where they can later graze on high protein diets. High protein diets have been shown to be a requirement to juvenile growth in various abalone species (Tayler, 1997; Mai et al., 1995b; Britz, 1996a; Coote et al., 2000). Unfortunately, data were not available to perform correlations between TAA and larval settlement and survival. Thus, it is not possible to ascertain if abalone larval settlement and survival respond to the same inductive processes as for attachment and metamorphosis. In addition, no significant correlations were found between EAA, NEAA, EAA/NEAA and *H. iris* attachment or metamorphosis, which indicates that such categories of amino acids are not involved in these larval processes. While no other studies have been conducted on the effect of EAA, NEAA, EAA/NEAA on abalone larval processes, Tayler (1997) found that these amino acid parameters do not affect abalone (*H. kamtschatkana*) growth.

2.5.2 Effect of biofilm age on abalone larvae

Matured biofilms clearly produced greater percent attachment, metamorphosis, settlement, and survival than young biofilms. Compared to younger biofilms, older biofilms have been found to enhance attachment (Roberts 2001b; Roberts et al. 2007), metamorphosis (Daume et al. 2004; Roberts et al., 2007), settlement (Moss and Tong, 1992; Daume et al., 2004), and survival (Daume et al., 2004) for different abalone species. Roberts et al. (2007) compared microalgal biofilms of different ages (5 days = young, > 25 days = old) for their ability to induce attachment and metamorphosis in *H. iris*, and found a significantly positive correlation between biofilm age and both of these larval processes. Daume et al. (2004) seeded *Ulva lens* to produce biofilms of different ages, and found that *H. rubra* improved settlement and survival rates by about 25-50% and 50%, respectively as the biofilm matured from 4 to 18 days. These studies support the notion that biofilms may produce more ECPs as they get older (more ECP-producing microalgal cells within the biofilm), and that these ECPs are responsible for signalling to larvae that their immediate environment has sufficient nutrients to support larval and juvenile live stages. ECPs have been shown to accumulate in biofilms over time (Batté et al., 2003). Previous studies also have shown that ECPs can stimulate larval settlement in marine invertebrates (Lam et al., 2005; Chen, 2007). For example, Chen (2007) extracted ECPs from various strains of benthic diatoms, and found a

significantly positive correlation between ECP amount and larval settlement for *H. diversicolor*, after a minimum ECP amount of 30 pg / 100 μm^2.

Results from the amino acid composition within young and matured biofilms in this study showed significantly greater amounts of several amino acids, TAA, % cover, and NEAA in matured compared to young biofilms. These results could be due to the accumulation of ECPs (which contain amino acids) during the growth phase of the microalgae within the biofilm. Alternatively, it also is possible that as the biofilm ages, it is colonized by bacteria, which may be responsible for the signalling cue (Chiu et al., 2007). Indeed, Roberts et al. (2007) suggested that bacteria provide an important role in the induction of *H. iris* metamorphosis, but may not be crucial in larval attachment. However, the abundance of bacteria was not monitored in this study, and it was assumed to be minimal and decreasing in density as the biofilms aged (Batté et al., 2003; Faimali et al., 2004). Thus, it still is unclear what specific components of an older biofilm are involved in the signalling mechanism, or how this mechanisms actually works. Further research may evaluate other compounds, such as fatty acids and non-protein nutrients as possible inducers of these larval and post-larval processes.

Figure 2. 1. Mean (±SE) percent (a) attachment, and (b) metamorphosis of abalone larvae within young and matured microalgal biofilms after 7 days, n = 3.

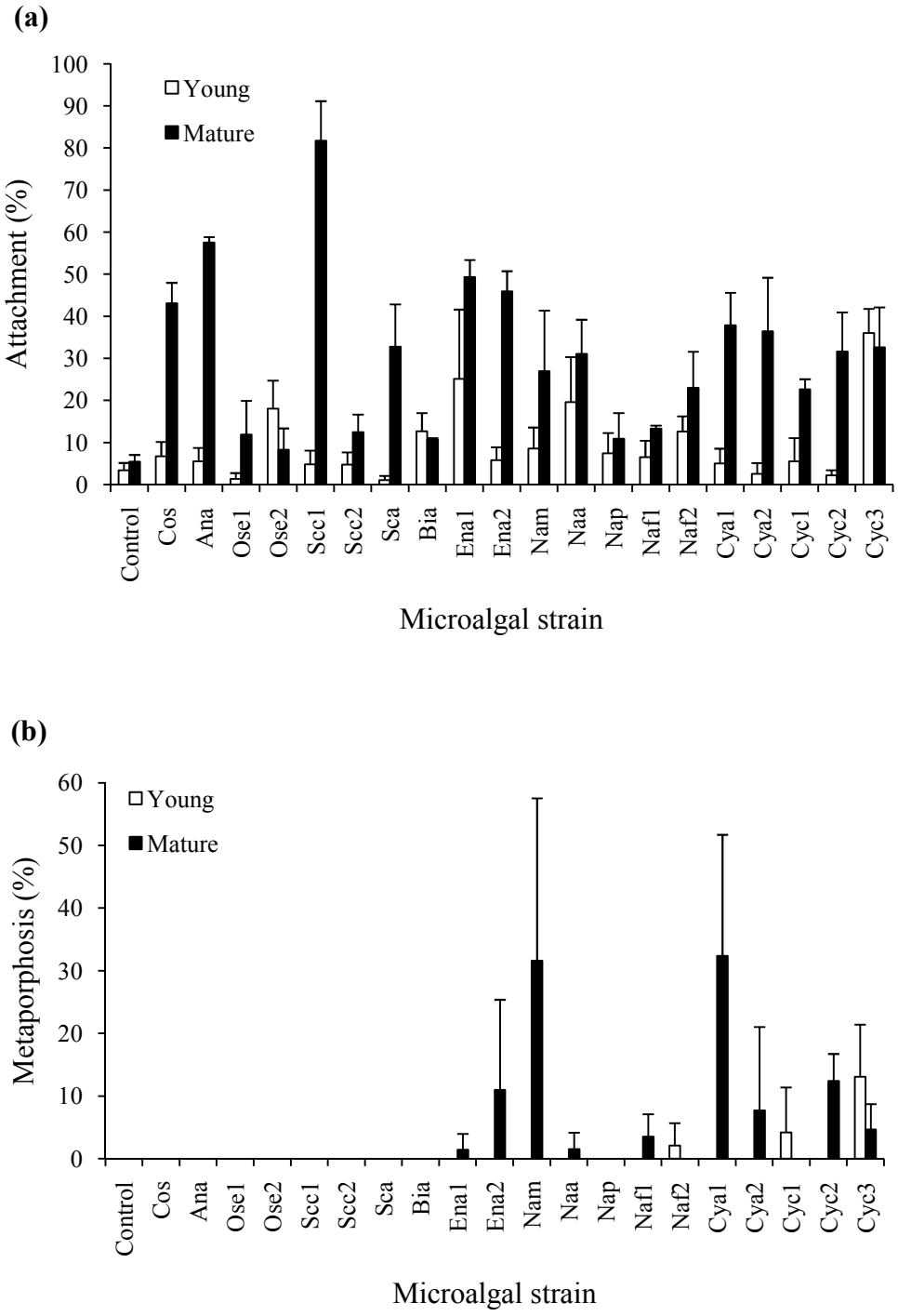


Figure 2. 2. Mean (±SE) percent (a) settlement, and (b) survival of abalone larvae within young and matured microalgal biofilms after 14 days, n = 3.

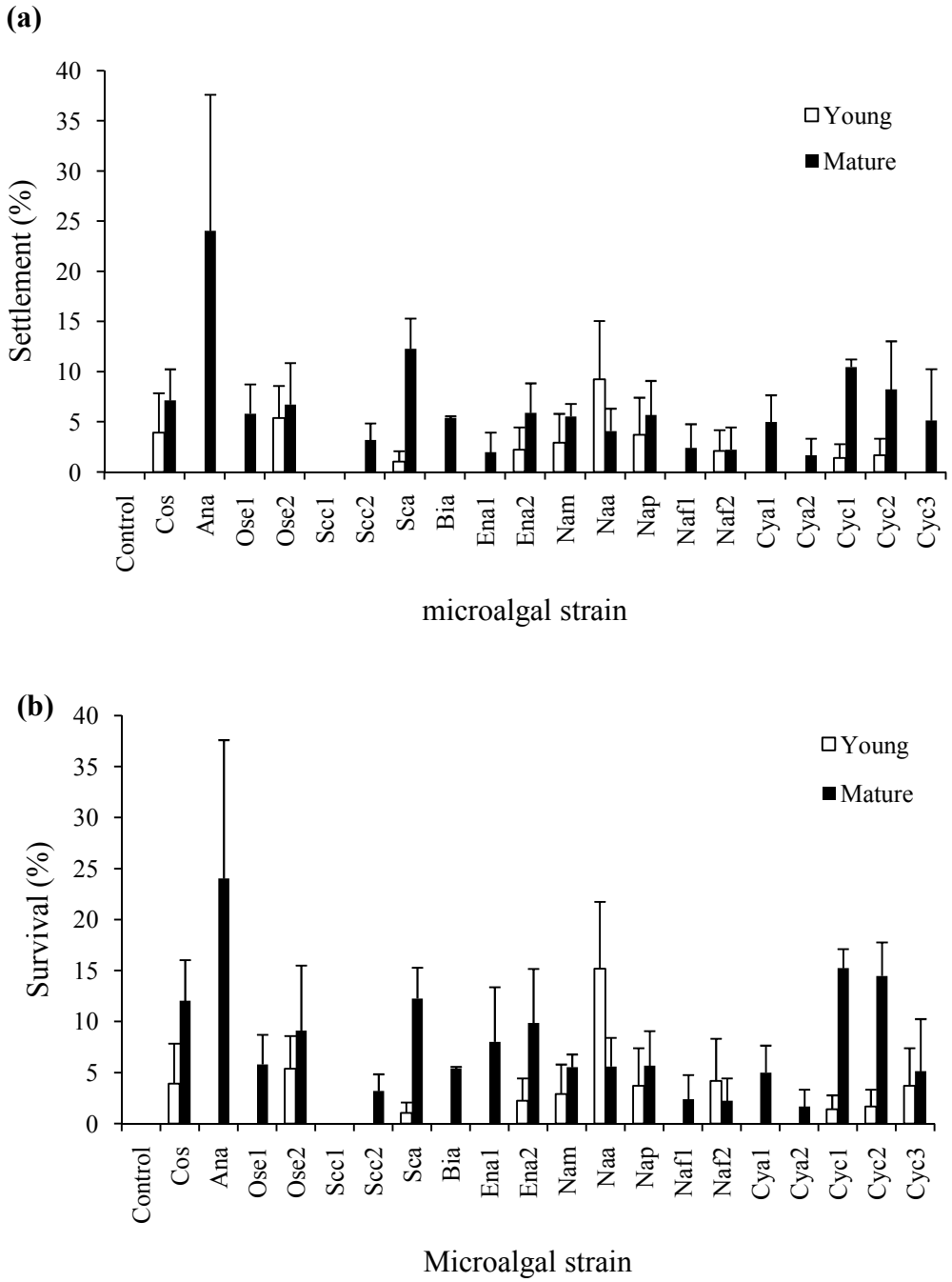


Figure 2. 3. Mean (±SE) percent biofilm cover of cultures with different microalgal strains after 7, 14, and 21 days, n = 3. The microalgal culturing times correspond to: 7 days = time 0 for the larval experiment, 14 days = 7 days for the larval experiment, and 21 days = 14 days for the larval experiment.

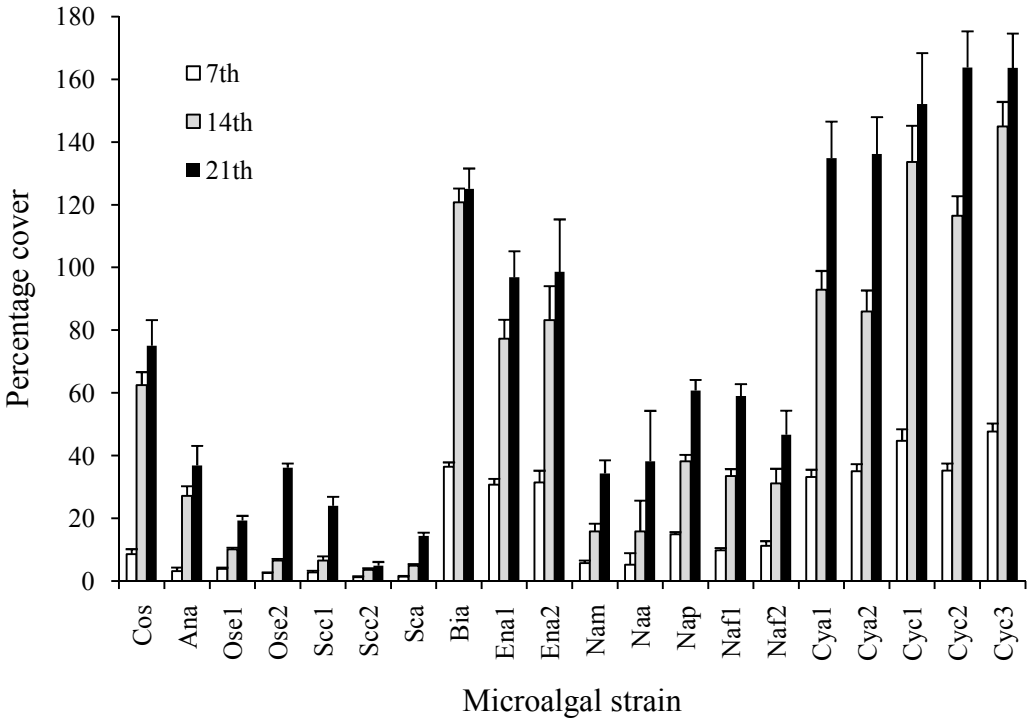


Table 2. 1. Characteristics (identification number, collection site, cell length and width) of the microalgal strains used in this study. R = rocky shore and S = sandy estuary

Family Species	ID	Site	length (μm)	Width (μm)
Chroococcaceae				
Coccochloris cf. *stagnin*	Cos	Devenport - (R)	24.0±7.6	14.9±5.0
Anacystis cf. *aeruginosa*	Ana	Devenport - (R)	16.8±2.3	11.9±3.2
Schizotrichaceae				
Schizothrix cf. *calcicola*	Scc1	Devenport - (R)	5.1±0.5	3.4±0.3
	Scc2	Point Chevalier - (S)	4.2±0.5	3.5±0.9
Schizothrix cf. *arenaria*	Sca	Devenport - (R)	7.9±2.3	2.2±0.3
Oscillatoriaceae				
Oscillatoria cf. *erythrata*	Ose1	Eastern Beach - (S)	3.3±1.3	1.7±0.3
	Ose2	Point Chevalier - (S)	3.1±0.6	2.1±0.5
Triceratiaceae				
Biddulphia cf. *aurita*	Bia	Devenport - (R)	88.5±8.7	28.6±1.6
Entomoneidaceae				
Entomoneis cf. *alata*	Ena1	Eastern Beach - (S)	52.9±1.7	20.9±0.7
	Ena2	Point Chevalier - (S)	51.9±2.0	18.2±2.5
Naviculaceae				
Navicula cf. *minuscule*	Nam	Bucklands Beach- (R)	32.2±1.3	9.3±0.9
Navicula cf. *atomoides*	Naa	Point Chevalier - (S)	12.0±0.5	6.7±0.9
Navicula cf. *falaisensis*	Naf1	Eastern Beach - (S)	29.0±4.3	9.3±0.8
	Naf2	Devenport - (R)	28.3±2.3	9.6±0.8
Navicula cf. *perminuta*	Nap	Bucklands Beach- (R)	36.1±2.3	9.5±3.4
Bacillaraceae				
Cylindrotheca cf. *acicularis*	Cya1	Bucklands Beach- (R)	57.9±1.7	7.9±1.0
	Cya2	Point Chevalier - (S)	67.7±2.8	8.0±1.0
Cylindrotheca cf. *closterium*	Cyc1	Eastern Beach - (S)	64.7±5.6	9.0±1.1
	Cyc2	Devenport - (R)	59.0±3.6	7.7±0.3
	Cyc3	Bucklands Beach- (R)	65.8±5.7	6.4±1.4

Table 2. 2. Kruskal-Wallis tests on larval attachment and metamorphosis at day 7 for different microalgal strains (fixed factor), and young and matured biofilms. All analyses were adjusted for ties, n = 3.

	Attachment						*Metamorphosis*					
	Young biofilms			Matured bioflims			Young biofilms			Matured bioflims		
Strain	Median	Rank	Z	Median	Rank	Z	Median	Rank	Z	Median	Rank	Z
Control	0.05	24.0	-0.77	0.04	6.7	-2.45	0.00	29.5	-0.24	0.00	23.0	-0.87
Cos	0.08	31.7	-0.03	0.48	44.8	1.24	0.00	29.5	-0.24	0.00	23.0	-0.87
Ana	0.05	28.8	-0.31	0.59	57.7	2.49	0.00	29.5	-0.24	0.00	23.0	-0.87
Scc1	0.00	15.7	-1.58	0.08	15.5	-1.60	0.00	29.5	-0.24	0.00	23.0	-0.87
Scc2	0.20	49.3	1.68	0.07	11.2	-2.02	0.00	29.5	-0.24	0.00	23.0	-0.87
Sca	0.03	26.0	-0.58	0.76	62.0	2.90	0.00	29.5	-0.24	0.00	23.0	-0.87
Ose1	0.04	26.3	-0.55	0.17	16.0	-1.55	0.00	29.5	-0.24	0.00	23.0	-0.87
Ose2	0.00	14.5	-1.69	0.29	37.8	0.56	0.00	29.5	-0.24	0.00	23.0	-0.87
Bia	0.13	44.8	1.24	0.11	12.8	-1.86	0.00	29.5	-0.24	0.00	23.0	-0.87
Ena1	0.19	42.8	1.05	0.52	50.7	1.81	0.00	29.5	-0.24	0.00	23.0	-0.87
Ena2	0.07	29.8	-0.21	0.50	47.5	1.50	0.00	29.5	-0.24	0.00	31.0	-0.10
Nam	0.08	34.3	0.23	0.15	29.5	-0.24	0.00	29.5	-0.24	0.06	44.3	1.19
Naa	0.15	45.3	1.29	0.23	35.0	0.29	0.00	29.5	-0.24	0.17	58.3	2.55
Naf1	0.06	31.5	-0.05	0.12	15.2	-1.63	0.00	29.5	-0.24	0.00	31.3	-0.06
NaF2	0.06	31.5	-0.05	0.14	17.0	-1.45	0.00	29.5	-0.24	0.00	23.0	-0.87
Nap	0.13	45.7	1.32	0.27	27.3	-0.45	0.00	39.7	0.74	0.04	40.0	0.77
Cya1	0.03	26.3	-0.55	0.33	43.0	1.07	0.00	40.3	-0.24	0.24	60.0	2.71
Cya2	0.00	19.7	-1.19	0.28	41.2	0.89	0.00	29.5	-0.24	0.00	35.0	0.29
Cyc1	0.00	24.2	-0.76	0.24	29.7	-0.23	0.00	40.3	0.81	0.00	23.0	-0.87
Cyc2	0.02	19.7	-1.19	0.25	36.0	0.39	0.00	29.5	-0.24	0.13	54.3	2.16
Cyc3	0.33	60.0	2.71	0.38	35.5	0.34	0.11	61.0	2.81	0.06	41.7	0.94
Overall		32.0			32.0			32.0			32.0	
	H = 26.34, df = 20, p = 0.155			H = 45.57, df = 20, p = 0.001			H = 43.95, df = 20, p = 0.002			H = 45.91, df = 20, p = 0.001		

Table 2. 3. Kruskal-Wallis test on larval attachment, metamorphosis at day 7, and settlement and survival at day 14 compared between young and matured microalgal biofilms (fixed factor). All analyses were adjusted for ties, n = 60.

	Attachment			*Metamorphosis*		
Biofilm	Median	Rank	Z	Median	Rank	Z
Young	0.06	43.3	-6.20	0.00	56.9	-2.03
Matured	0.25	83.7	6.20	0.00	70.0	2.03
Overall		63.5			63.5	
	H = 38.67, df = 1, p = 0.000			H = 9.10, df = 1, p = 0.003		
	Settlement			*Survival*		
Biofilm	Median	Rank	Z	Median	Rank	Z
Young	0.00	50.2	-4.09	0.00	49.0	-4.45
Matured	0.05	76.8	4.09	0.06	78.0	4.45
Overall		63.5			63.5	
	H = 24.89,df = 1, p = 0.000			H = 21.45, df = 1, p = 0.000		

Table 2. 4. Kruskal-Wallis tests on abalone settlement and survival at day 14 for different microalgal strains (fixed factor), and young and matured biofilms. All analyses were adjusted for ties, n = 3.

	Settlement						*Survival*					
	Young biofilms			Matured bioflims			Young biofilms			Matured bioflims		
Strain	Median	Rank	Z	Median	Rank	Z	Median	Rank	Z	Median	Rank	Z
Control	0.00	26.0	-0.58	0.00	13.0	-1.84	0.00	26.0	-0.58	0.00	12.0	-1.94
Cos	0.00	38.0	0.58	0.04	37.7	0.55	0.00	26.0	-0.58	0.09	47.7	1.52
Ana	0.00	26.0	-0.58	0.25	46.0	1.36	0.00	26.0	-0.58	0.25	45.7	1.32
Scc1	0.00	26.0	-0.58	0.08	36.8	0.47	0.00	26.0	-0.58	0.08	33.7	0.16
Scc2	0.05	47.5	1.45	0.06	36.7	0.45	0.05	46.5	1.40	0.05	35.7	0.36
Sca	0.00	26.0	-0.58	0.00	13.0	-1.84	0.00	26.0	-0.58	0.00	12.0	-1.94
Ose1	0.00	26.0	-0.58	0.04	25.5	-0..63	0.00	26.0	-0.58	0.04	22.5	-0.92
Ose2	0.00	34.7	0.26	0.12	53.2	2.05	0.00	26.0	-0.58	0.12	47.8	1.53
Bia	0.00	26.0	-0.58	0.06	34.2	0.21	0.00	26.0	-0.58	0.06	29.2	-0.27
Ena1	0.00	26.0	-0.58	0.00	21.7	-1.00	0.00	26.0	-0.58	0.06	34.3	0.23
Ena2	0.00	36.3	0.42	0.09	37.2	0.50	0.00	36.0	0.39	0.11	39.0	0.68
Nam	0.00	37.0	0.48	0.06	35.3	0.32	0.00	36.3	0.42	0.06	31.7	-0.03
Naa	0.08	49.0	1.65	0.05	29.2	-0.27	0.15	59.0	2.61	0.08	32.8	0.08
Naf1	0.00	37.5	0.53	0.05	33.5	0.15	0.00	37.0	0.48	0.05	29.8	-0.21
NaF2	0.00	26.0	-0.58	0.00	22.8	-0.89	0.00	26.0	-0.58	0.00	20.5	-1.11
Nap	0.00	36.0	0.39	0.00	22.3	-0.94	0.00	37.7	0.55	0.00	20.0	-1.16
Cya1	0.00	26.0	-0.58	0.06	34.2	0.21	0.00	26.0	-0.58	0.06	30.7	-0.13
Cya2	0.00	26.0	-0.58	0.00	19.2	-1.24	0.00	26.0	-0.58	0.00	16.8	-1.47
Cyc1	0.00	35.0	0.29	0.11	52.7	2.00	0.00	35.0	0.29	0.16	53.2	2.05
Cyc2	0.00	35.5	0.34	0.08	39.7	0.74	0.00	35.5	0.34	0.17	51.7	1.90
Cyc3	0.00	26.0	-0.58	0.00	28.3	-0..36	0.00	37.0	0.48	0.00	25.3	-0.65
Overall		32.0			32.0			32.0			32.0	
	H = 20.24, df = 20, p = 0.443			H = 23.74, df = 20, p = 0.254			H = 28.71, df = 20, p = 0.094			H = 28.59, df = 20, p = 0.096		

Table 2. 5. Amino acid profiles for microalgal strains in young biofilms (7 days old). The amount of each amino acid is presented as the mean of three replicates, with units of mg/100mg for each amino acid sample. Total amino acids (TAA) are shown as µg/cell. Percent biofilm cover, essential amino acids (EAA), and non-essential amino acids (NEAA) also are shown.

Strain	Cos	Ana	Ose1	Ose2	Scc1	Scc2	Sca	Bia	Ena1	Ena2	Nam	Naa	Nap	Naf1	Naf2	Cya1	Cya2	Cyc1	Cyc2	Cyc3
Asx	10.28	10.20	8.57	4.91	5.48	6.81	8.46	7.19	8.09	7.47	7.32	6.10	6.20	8.45	8.99	4.59	3.69	8.19	6.73	9.42
Glx	11.28	11.58	12.23	6.48	9.00	7.90	13.14	8.07	10.05	8.50	9.28	7.37	7.03	8.48	9.04	4.99	4.40	8.75	7.43	9.06
Ser	15.63	15.57	16.48	16.94	16.14	27.82	16.27	23.72	15.37	12.52	9.97	15.93	15.15	13.19	15.17	14.09	13.07	15.48	14.81	16.22
His	5.10	4.10	1.93	6.16	2.53	2.93	1.73	3.47	1.95	2.61	4.83	5.13	4.34	2.10	2.33	3.06	2.28	2.78	2.67	1.12
Gly	2.86	3.12	2.19	4.85	6.33	5.46	3.96	6.77	4.25	4.64	2.22	6.81	5.62	2.21	7.89	5.95	2.01	5.35	6.68	0.45
Thr	9.89	9.88	10.67	1.32	10.01	8.70	10.73	9.59	9.84	9.18	8.21	9.26	9.96	10.96	10.35	11.40	14.80	9.89	9.30	11.31
Ala+Tau	8.18	7.94	9.94	16.22	18.20	4.50	9.84	6.47	11.55	22.19	15.54	11.61	15.00	20.23	11.89	16.35	18.06	14.19	15.78	12.66
Arg	2.69	2.72	2.71	3.69	2.63	2.37	2.90	2.40	2.71	2.30	1.80	2.74	2.41	2.30	2.74	2.94	2.76	2.55	2.66	2.08
GABA	0.01	0.00	0.07	0.00	0.00	0.00	0.08	0.11	0.00	0.00	0.00	0.00	0.04	0.00	0.06	0.05	0.06	0.11	0.04	0.08
Tyr	2.71	2.65	2.89	3.85	2.59	2.35	3.19	2.34	2.87	2.56	19.62	2.78	2.82	2.97	2.94	3.11	4.46	2.85	2.45	3.10
Met	0.59	0.61	0.84	1.20	1.32	1.04	0.83	0.96	1.17	0.18	0.42	0.96	0.14	1.86	1.52	1.30	2.97	0.42	2.00	1.68
Val	5.58	5.50	5.42	6.19	4.08	5.03	5.38	4.40	4.90	3.95	3.94	4.16	4.15	5.32	4.93	4.56	7.07	4.43	4.03	6.28
Phe	8.13	8.48	7.83	7.99	5.76	5.92	6.58	7.17	8.58	7.23	5.87	7.71	7.81	7.94	7.13	8.53	9.83	7.68	7.37	8.95
Ile	4.24	4.78	4.73	4.54	2.26	6.01	4.12	3.02	4.14	3.16	2.52	3.41	3.23	3.93	3.90	3.41	5.32	3.64	3.23	4.45
Leu	10.66	11.38	11.09	12.00	7.73	7.83	10.07	9.53	10.48	8.73	7.03	9.73	9.70	8.88	8.48	9.13	8.67	9.18	9.20	10.83
Orn	0.00	0.01	0.01	0.00	0.01	0.03	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Lys	2.17	1.50	2.39	3.67	5.95	5.31	2.73	4.79	4.04	4.78	1.41	6.33	6.39	1.17	2.62	6.55	0.57	4.52	5.59	2.31
Pro	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TAA	0.22	0.27	0.02	0.07	0.05	0.003	0.02	0.13	0.30	0.21	0.42	0.15	0.17	0.31	0.20	0.25	0.33	0.11	0.26	0.07
% cover	8.64	3.14	3.90	2.59	2.73	1.27	1.45	36.48	30.70	31.43	5.71	5.27	14.92	9.79	11.19	33.16	35.01	44.68	35.24	47.66
EAA (%)	49.04	48.95	47.6	46.76	42.26	45.13	45.06	45.32	47.82	42.11	36.05	49.41	48.13	44.46	44.01	50.88	54.26	45.09	46.07	49.01
NEAA (%)	50.96	51.05	52.38	53.24	57.74	54.87	54.94	54.68	52.18	57.89	63.95	50.59	51.87	55.54	55.99	49.12	45.74	54.91	53.93	50.99
EAA/NEAA	0.96	0.96	0.91	0.88	0.73	0.82	0.82	0.83	0.92	0.73	0.56	0.98	0.93	0.80	0.79	1.04	1.19	0.82	0.85	0.96

Table 2. 6. Amino acid profiles for microalgal strains in matured biofilms (14 days old). The amount of each amino acid is presented as the mean of three replicates, with units of mg/100mg for each amino acid sample. Total amino acids (TAA) are shown as µg /cell. Percent biofilm cover, essential amino acids (EAA), and non-essential amino acids (NEAA) also are shown.

Strain	Cos	Ana	Ose1	Ose2	Scc1	Scc2	Sca	Bia	Ena1	Ena2	Nam	Naa	Nap	Naf1	Naf2	Cya1	Cya2	Cyc1	Cyc2	Cyc3
Asx	10.10	9.87	8.35	4.89	5.32	6.06	8.18	6.56	7.90	7.53	7.81	5.93	6.11	8.52	8.47	4.50	3.68	7.96	6.54	9.39
Glx	10.03	10.15	10.79	5.84	7.89	6.36	11.49	6.66	8.89	7.74	8.97	6.48	6.27	7.73	7.71	4.43	3.98	7.70	6.53	8.16
Ser	19.43	19.0	20.34	21.38	19.82	31.36	19.91	27.43	19.03	15.97	13.48	19.61	18.90	16.85	18.12	17.49	16.55	19.06	18.22	20.46
His	3.19	2.53	1.20	3.91	1.56	1.66	1.07	2.01	1.22	1.67	3.28	3.18b	2.72	1.35	1.40	1.91	1.45	1.72	1.65	0.71
Gly	4.94	5.31	3.75	8.50	10.79	8.54	6.73	10.86	7.30	8.23	4.16	11.64	9.73	3.92	13.09	10.25	3.53	9.15	11.42	0.79
Thr	10.85	10.69	11.62	1.47	10.85	8.66	11.59	9.79	10.75	10.33	9.80	10.06	10.96	12.35	10.91	12.49	16.53	10.74	10.10	12.59
Ala+Tau	6.13	5.87	7.40	12.35	13.49	3.06	7.27	4.51	8.62	17.08	12.68	8.63	11.29	15.59	8.57	12.24	13.79	10.55	11.71	9.63
Arg	3.43	3.41	3.42	4.78	3.31	2.74	3.64	2.84	3.43	3.01	2.49	3.45	3.08	3.01	3.36	3.75	3.58	3.22	3.35	2.69
GABA	0.01	0.00	0.09	0.01	0.00	0.00	0.09	0.13	0.00	0.00	0.00	0.00	0.05	0.00	0.07	0.06	0.07	0.13	0.06	0.11
Tyr	1.96	1.88	2.07	2.82	1.84	1.54	2.26	1.57	2.06	1.89	15.39	1.99	2.04	2.20	2.04	2.24	3.27	2.04	1.75	2.27
Met	0.52	0.53	0.73	1.07	1.14	0.83	0.71	0.78	1.02	0.16	0.40	0.83	0.12	1.67	1.27	1.14	2.65	0.36	1.73	1.49
Val	6.22	6.05	6.01	7.01	4.49	5.09	5.91	4.56	5.44	4.52	4.78	4.59	4.65	6.09	5.28	5.08	8.03	4.89	4.45	7.11
Phe	6.44	6.62	6.15	6.41	4.50	4.24	5.12	5.27	6.76	5.87	5.05	6.04	6.20	6.45	5.42	6.74	7.91	6.02	5.77	7.18
Ieu	4.22	4.69	4.68	4.59	2.22	5.42	4.04	2.80	4.11	3.23	2.73	3.36	3.23	4.02	3.73	3.39	5.39	3.59	3.19	4.50
Leu	10.62	11.18	10.97	12.13	7.61	7.07	9.88	8.82	10.39	8.93	7.62	9.60	9.69	9.09	8.11	9.09	8.79	9.06	9.07	10.95
Orn	0.36	1.06	0.75	0.19	0.96	3.94	0.18	2.21	0.18	0.34	0.24	0.14	0.37	0.29	0.65	0.54	0.37	0.59	0.49	0.29
Lys	1.55	1.06	1.70	2.66	4.20	3.44	1.92	3.19	2.88	3.51	1.10	4.48	4.58	0.86	1.80	4.68	0.42	3.20	3.96	1.68
Pro	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TAA	0.31	0.31	0.07	0.29	0.20	0.01	0.05	0.39	1.20	0.75	1.51	0.49	0.68	0.89	0.73	0.89	1.36	0.35	0.79	0.23
% cover	62.53	27.19	10.16	6.53	6.55	3.55	4.91	120.78	77.25	83.19	15.80	15.81	38.23	33.46	31.20	92.89	85.91	133.60	116.53	144.99
EAA (%)	47.04	46.76	46.47	44.03	39.89	39.14	43.88	40.06	46.00	41.22	37.27	45.59	45.24	44.89	41.29	48.25	54.75	42.82	43.28	48.90
NEAA (%)	52.96	53.24	53.53	55.97	60.11	60.86	56.12	59.94	54.00	58.78	62.73	54.41	54.76	55.11	58.71	51.75	45.25	57.18	56.72	51.10
EAA/NEAA	0.89	0.88	0.87	0.79	0.66	0.64	0.78	0.67	0.85	0.70	0.59	0.84	0.83	0.81	0.70	0.93	1.21	0.75	0.76	0.96

Table 2. 7. Kruskal-Wallis test on individual amino acid amounts (fixed factor) within young and matured biofilms. Bold numbers denote significant differences ($p < 0.05$).

	Young biofilms			Matured biofilms			H	P-value
Amino acid	Median	Rank	Z	Median	Rank	Z		
Asx	7.41	62.4	0.59	7.71	58.6	-0.59	0.35	0.553
Glx	8.70	69.3	2.76	7.72	51.8	-2.76	7.59	**0.006**
Ser	15.53	39.2	-6.70	19.11	81.8	6.70	44.85	**0.000**
His	2.79	77.2	5.26	1.70	43.8	-5.26	27.66	**0.000**
Gly	4.75	44.2	-5.14	8.15	76.8	5.14	26.46	**0.000**
Thr	9.97	49.3	-3.54	10.87	71.7	3.54	12.51	**0.000**
Ala+Tau	13.57	72.7	3.83	10.16	48.3	-3.83	14.68	**0.000**
Arg	2.64	41.1	-6.10	3.30	79.9	6.10	37.26	**0.000**
GABA	0.03	56.9	-1.14	0.03	64.1	1.14	1.32	0.251
Tyr	2.86	83.8	7.33	2.04	37.2	-7.33	53.76	**0.000**
Met	0.98	64.5	1.26	0.84	56.5	-1.26	1.60	0.206
Val	4.83	51.3	-2.91	5.20	69.7	2.91	8.45	**0.004**
Phe	7.82	81.7	6.68	6.04	39.3	-6.68	44.64	**0.000**
Ile	3.88	61.3	0.24	3.77	59.7	-0.24	0.06	0.809
Leu	9.20	61.4	0.27	9.08	59.6	-0.27	0.07	0.785
Orn	0.00	30.5	-9.45	0.39	90.5	9.45	89.26	**0.000**
Lys	3.69	70.3	3.09	2.66	50.7	-3.09	9.52	**0.002**
Pro	0.00	36.8	-7.45	0.00	84.2	7.45	55.55	**0.000**
TAA	0.17	42.7	-5.62	0.44	78.3	5.62	31.54	**0.000**
% cover	22.81	14.5	-2.04	35.85	21.7	2.04	4.15	**0.042**
EAA	46.41	70.7	3.22	44.44	50.3	-3.22	10.35	**0.001**
NEAA	53.59	50.3	-3.22	55.56	70.7	3.22	10.35	**0.001**
EAA/NEAA	0.87	70.7	3.22	0.80	50.3	-3.22	10.35	**0.001**

Table 2. 8. Pearson's correlations between larval parameters (percent attachment, metamorphosis, settlement and survival) and microalgal amino acid contents (TAA = total amino acids, EAA = essential amino acids, NEAA = non-essential amino acids, EAA/NEAA ratio, and percent biofilm cover. Bold numbers denote significant differences ($p < 0.05$). Correlation coefficients (R) and p-values (p) are shown. ND = values not detected.

	Attachment		*Metamorphosis*		*Settlement*		*Survival*	
	R	p	R	p	R	p	R	p
TAA	0.322	**0.000**	0.514	**0.000**	ND	ND	ND	ND
% cover	0.285	**0.002**	0.255	**0.005**	0.138	0.133	0.223	**0.014**
EAA	-0.075	0.413	-0.057	0.534	ND	ND	ND	ND
NEAA	0.075	0.413	0.057	0.534	ND	ND	ND	ND
EAA/NEAA	-0.072	0.434	-0.038	0.684	ND	ND	ND	ND

Chapter 3 Effects of dual microalgal species biofilms on New Zealand black-footed abalone (*Hailotis iris*) larval/post-larval processes

3.1 Abstract

Dual microalgal species biofilms with distinct amino acid characteristics were tested for their effects on abalone (*Haliotis iris*) initial attachment, metamorphosis, settlement and survival. Nine microalgal species, isolated from around Auckland, New Zealand, were grown in the laboratory, and used to produce nine dual microalgal species biofilms. Abalone larvae were exposed to the different biofilm treatments and controls (no biofilms) for 7 (attachment and metamorphosis) and 14 (settlement and survival) days. The larvae performed significantly better in the dual microalgal biofilms compared to controls, and some biofilms resulted in better attachment, metamorphosis, settlement, and survival than others. For example, the best-performing biofilms almost always contained cyanobacteria (*Oscillatoria* cf. *erythrata*, *Anacystis* cf. *aeruginosa*, and *Schizothrix* cf. *calcicola*), which are attributed with better nutritional values. However, the amino acid profiles did not produce a clear pattern with regard to their effects on the four larval processes. Instead, total amino acid (TAA) content was positively correlated with percent attachment and metamorphosis. Greater TAA contents are likely to reflect greater amounts of extra-cellular polymers within the biofilms, which are suggested to improve larval performance.

3.2 Introduction

One of the most important stages in the life cycle of broadcast spawners is the transformation process from planktonic to benthic living. This life style transformation revolves around metamorphosis and settlement processes, which include dramatic morphological, chemical, and behavioral changes. As a consequence, larvae appear to be extremely sensitive to the physical and biological properties of their surrounding environment. This high receptivity to substrate characteristics allows larvae to assess the suitability (e.g., nutrient availability, level of physical stress) of the benthic site before and during settlement. For example, chemical signals from biofilms and their surface topography may cue larvae to choose to settle or move away from that substrate (Wieczorek and Todd 1998; Huggett et al., 2006). It is well-known that specific biofilms may promote settlement of a variety of invertebrate species, such as polychaetes (Pawlik 1992; Wieczorek and Todd,

1998), gastropods (Gordon et al. 2004, 2006), bivalves (Fitt et al., 2005), bryozoans (Mihm et al., 1981), and echinoderms (Johnson et al., 1991a, b; Huggett et al., 2006). In the marine environment, biofilms are formed by a complex matrix of molecules (e.g., amino acids, glycoproteins, humic materials; D'Souza et al., 2005; Karatan and Watnick, 2009) housing a range of bacteria, protozoans, fungi, and microalgae (Faimali et al., 2004). In addition, these biofilms often contain more than one dominant microalgal species, which may have different inductive properties for invertebrate larvae (Faimali et al., 2004). In contrast, hatchery conditions often regulate the biofilm community to one or two dominant microalgal species, which are cultured specifically to feed the target commercial species. In the case of abalone, these microalgal biofilms serve as food for the developing post-larvae (Uki 1995). Thus, it is of crucial importance to understand the function and the role of each component (e.g., bacteria, microalgae) in the culturing biofilm. Many studies have focused on the effects of a single-species biofilms, such as bacteria (Maki et al., 1990; Morse et al., 1992) and diatoms (Kawamura and Takami, 1995; Kawamura et al., 1996; Roberts 2001a, b), on invertebrate larvae. However, biofilms containing a mixture of species often produce better-performing larvae/post-larvae (Wieczorek and Todd, 1998, Daume et al., 2003). It is likely that this is due to the nutritional content of the biofilm, which may achieve a better nutritional balance when more than one species is involved (Gordon et al., 2006). Conversely, antagonistic interactions among species in the biofilm matrix may deter or hinder the physiological activities of larvae/post-larvae (Wieczorek and Todd, 1998; Huang and Boney, 1985).

In abalone aquaculture, single strain micro-algal films have been tested and shown to have a wide range of results for larval settlement (Kawamura et al., 1998; Roberts 2001a, b), and post-larval grazing (Daham et al., 2003). However, mixed microalgal biofilms and diets generally have better effects on larval culture (Bryan and Qian, 1998; Gordon et al., 2006). In fact, a mixture of *Navicula incerta*, *Amphiprora paludosa*, and *Nitzschia thermalis* was found to produce higher feed conversion efficiencies, growth, and survival of green abalone *H. fulgens* post-larvae (Viana et al., 2007). In addition, Gordon et al. (2006) found that a mixture of diatoms (*Nitzschia laevis, Amphora luciae*, and *Navicula* cf. *lenzii*) was a superior diet for *H. discus hannai* post-larval development compared to single-species diets, and this dietary improvement was attributed to a more balanced nutritional content within the biofilm. For the New Zealand black-footed abalone (*H. iris*), initial investigations have been made with regard to larval settlement and grazing on single-species microalgal biofilms (Kawamura et al., 1998; Roberts 2001a, b; Roberts et al., 2007). Roberts et al. (2007) tested a range of diatoms in

single-species biofilms and found improved initial *H. iris* attachment, but little consistent improvement in metamorphosis within 4-day experiments. In addition, Kawamura et al., (1998) found that *H. iris* post-larvae grew faster and survived better on two separate diatoms (*Achnanthes longipes*-1 and *Nitzschia* sp.), which were easily digested by abalone post-larvae. However, the effect of multi-species biofilms has not been studied for the early life history processes of *H. iris*. Thus, the aim of this study is to investigate the effects of dual microalgal species biofilms on black-footed abalone (*H. iris*) initial attachment, metamorphosis, settlement, and survival.

3.3 Materials and Methods

3.3.1 Micro-algal collection, isolation, and culture

Nine microalgal species were collected and isolated from coastal areas around Auckland Harbour, North Island, New Zealand (36°51'S, 174° 47'E), during low tide periods between June and September 2006. The nine microalgal species were *Anacystis* cf. *aeruginosa* (Ana), *Oscillatoria* cf. *erythrata* (Ose), *Schizothrix* cf. *calcicola* (Scc), *Biddulphia* cf. *aurita* (Bia), *Entomoneis* cf. *alata* (Ena), *Navicula* cf. *atomoides* (Naa), *Navicula* cf. *perminuta* (Nap), *Cylindrotheca* cf. *acicularis* (Cya), and *Cylindrotheca* cf. *closterium*(Cyc). After isolation, the microalgae were cultured in 20 mL F_2 media for 14 days under a 1200 lux light source with 12 light/12 dark cycles at a constant temperature (15±1°C). These mono-specific cultures were used to produce 36 pairs of dual microalgal species biofilms. This was accomplished by adding a 1 mL volume of each of two different microalgal species and placing the mixture into Petri dishes. Then, F_2 media (Guillard and Ryther, 1962; Guillard, 1975) was added to make up 20 mL solutions for three replicates of each of the 36 distinct dual mixtures (treatments). The plates were left for 7 days to develop biofilms.

3.3.2 Amino acid profiles

The amino acid composition of each dual microalgal culture was determined by scraping the microalgal cells from the bottom of a different set of Petri dishes where biofilms were allowed to develop for 14 days. All samples were placed inside individual 25 mL plastic test tubes with lids, and centrifuged at 2000g for 10 minutes. The amino acid analysis was

conducted in accordance with Paramás et al. (2006). The microalgal precipitates were mixed with 5 mL of water and a 5 mL 13 M concentrate of hydrochloric acid with 0.1% phenol solution. The samples were heated in a Velp digester at 110 °C for 12 hrs under nitrogen gas. After cooling, the solution was adjusted to a pH of 6.5-7.5 by adding 1 M NaOH solution. The final volume was recorded and stored in a -20 °C freezer until further analysis.

In preparation for the HPLC, the samples were reacted with o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (MCE) at room temperature to induce fluorescence. The OPA solution was made with 500 mg of o-phthaldialdehyde (Merck) in 22.5 mL of ethanol and 0.4 M borate buffer (pH 10) and 400 µL of 2-mercaptoethanol (Sigma) to a total volume of 25 mL. A 50 µL volume of each sample was mixed with 450 µL sodium phosphate buffer (100 mM, pH 7.3; BDH), 100 µL OPA reagent, and 50 µL of an internal standard (3, 5-dibromotyrosine) in a new 1.5 mL vial. The final internal standard concentration in the sample solution was $1x10^{-5}$ M. A high-pressure liquid chromatography (HPLC) system was used to analyze the samples, after calibration with a mixed L-amino acid standard. The HPLC was attached to a Shimadzu LC-10AD automatic sampler fitted with a DGU-2A helium de-gas system. The solid phase consisted of a NOVA-PAK® C_{18} column (4 µm 3.9 × 150 mm, Waters). The mobile phase was adapted from Paramás et al. (2006) to provide a gradient elution. The first mobile phase was 80:19:1 ratio of sodium phosphate buffer (10 mM, pH 7.3)/methanol/tetrahydrofurane (solution A). The second mobile phase contained a 20:80 sodium phosphate buffer (10 mM, pH 7.3)/methanol (solution B). Solution A was added at 100% at 0.1 mL/min for the first 3 min, after which time the flow rate was increased to 1.5 mL/min. At 14.5 min, the gradual addition of solution B was started. At 19.5 min, the ratio of solution A to B was 85:15. At 24.5 and 45 min, the ratios were 70:30 and 30:70, solution A to B, respectively. Fluorometric detection of amino acids was obtained at excitation and emission wavelengths of 340 and 426 nm, respectively. Detection of proline was obtained through visible spectroscopy (Ultrospec 21000pro UV/Visible) at a 517 nm wavelength of each sample prior to pre-treatment with OPA, according to Amerine and Ough (1980).

A cell count for each dual microalgal culture was used to standardize individual amino acid weights per cell to the total amino acid (TAA). Distinction between essential amino acids (EAA) and non-essential amino acids (NEAA) was made following Allen and Kilgore (1975).

EAA:NEAA ratios were calculated by dividing the total EAA weight by the total NEAA weight.

3.3.3 Larval settlement experiments

H. iris larvae of about 7- 8 days old were obtained from OceaNZ Blue Ltd., at Bream Bay Aquaculture Park, northern New Zealand, and transported to the AUT lab in filtered seawater (Milipore™, 0.45 μm). Immediately after arrival, 25-35 larvae together with 5 mL filtered seawater were put into the experimental Petri dishes containing the dual microalgal species biofilms. The same larval density was added to each of three controls (no microalgae added). Larval attachment (initial attachment to the substrate, but cilia still visible) and metamorphosis (appearance of cephalic tentacles and complete disappearance of cilia) were recorded after 7 days. After day 14, settlement (sustained attachment to the substrate, peristomal shell starts to form and grow on the right side of the animal) and survival were recorded.

3.3.4 Statistical analyses

Kruskal-Wallis tests were performed on percent attachment, metamorphosis, settlement, and survival data (dual microalgal biofilm as a fixed factor). Multiple comparisons were obtained with individual Dunn's multiple comparison tests. Pearson correlations were performed between percent attachment and metamorphosis with TAA, EAA, NEAA, and EAA/NEAA ratios. All the analyses were performed using MINITAB version 14 software package.

3.4 Results

3.4.1 Larval attachment after 7 days

Larval attachment after 7 days of exposure to dual microalgal biofilms ranged between 15-70 % for all treatments (Fig. 3.1a). These values were significantly higher than the 5% attachment observed in the control plates (Fig. 3.1a; Table 3.1). Among the 36 dual

macroalgal biofilm treatments, five biofilms showed significantly higher attachment (over about 60%) than other biofilms (Dunn's multiple comparison tests; $p < 0.001$). These biofilms were composed of the microalgal pairs *Oscillatoria* cf. *erythrata* (Ose) + *Cylindrotheca* cf. *acicularis* (Cya) at 61.05±10.05%, *Oscillatoria* cf. *erythrata* (Ose) + *Cylindrotheca* cf. *closterium* (Cyc) at 61.67±14.37%, *Anacystis* cf. *aeruginosa* (Ana) + *Navicula* cf. *perminuta* (Nap) at 63.29±11.80%, *Schizothrix* cf. *calcicola* (Scc) + *Cylindrotheca* cf. *acicularis* (Cya) at 59.54±2.47%, and *Schizothrix* cf. *calcicola* (Scc) + *Cylindrotheca* cf. *closterium* (Cyc) at 61.25±10.48%.

3.4.2 Larval metamorphosis after 7 days

The percent metamorphosis after 7 days of exposure to the dual microalgal biofilms was relatively low (less than about 35%; Fig. 3.1b). From the 36 treatments, there were 14 that resulted in significantly higher metamorphosis than the control (Fig. 3.1b; Table 3.1). Dunn's multiple comparison tests indicated that *Oscillatoria* cf. *erythrata* (Ose) + *Cylindrotheca* cf. *acicularis* (Cya) at 29.16±4.92%, *Anacystis* cf. *aeruginosa* (Ana) + *Navicula* cf. *perminuta* (Nap) at 32.45±13.42%, and *Biddulphia* cf. *aurita* (Bia) + *Cylindrotheca* cf. *closterium* (Cyc) at 34.20±9.99% resulted in significantly greater percent metamorphosis compared to the other treatments and controls.

3.4.3 Larval settlement after 14 days

Larval settlement after 14 days was extremely low (less than 10%), except for the *Entomoneis* cf. *alata* (Ena) + *Cylindrotheca* cf. *closterium* (Cyc) microalgal biofilm, which achieved a 11.83±1.65% settlement (Fig. 3.2a; Table 3.2). In addition, control plates did not have any larval settlement at all. In general, biofilms containing *Cylindrotheca* cf. *acicularis* (Cya) and *Cylindrotheca* cf. *closterium* (Cyc) resulted in slightly better settlement than other treatments and controls.

3.4.4 Larval survival after 14 days

Larval survival after 14 day of exposure to the biofilms was relatively low (less than 32%) for all 36 treatments, and no survival was observed in the control plates (Fig. 3.2b; Table 3.2). *Entomoneis* cf. *alata* (Ena) + *Cylindrotheca* cf. *closterium* (Cyc) at 28.61±6.33% and *Schizothrix* cf. *calcicola* (Scc) + *Cylindrotheca* cf. *closterium* (Cyc) at 31.57±4.16% had significantly better larval survival than the other treatments (Dunn's multiple comparison tests; $p < 0.001$). Five other treatments had larval survivals between 20-25%. These included *Oscillatoria* cf. *erythrata* (Ose) + *Cylindrotheca* cf. *acicularis* (Cya) at 23.10±5.85%, *Anacystis* cf. *aeruginosa* (Ana) + *Navicula* cf. *perminuta* (Nap) at 21.67±9.19%, *Cylindrotheca* cf. *acicularis* (Cya), *Cylindrotheca* cf. *closterium* (Cyc) at 23.24±11.90%, and *Navicula* cf. *atomoides* (Naa) + *Cylindrotheca* cf. *acicularis* (Cya) at 22.84±4.04%.

3.4.5 Amino acid profiles and correlations with larval parameters

In general, the dual microalgal biofilms contained distinct amino acid profiles, and generally were high in Glx, Thr, Ala+ Tau, and Leu concentrations and low GABA, Meth, Orn, and Pro concentrations. Total amino acid (TAA) concentrations ranged from 0.07 to 0.30µg/cell. EAA proportions ranged from 49.15% (*Biddulphia* cf. *aurita* +*Schizothrix* cf. *calcicola*) to 67.96% (*Navicula* cf. *perminuta*+ *Cylindrotheca* cf. *closterium*), and NEAA proportions ranged from 32.04% (*Navicula* cf. *perminuta*+ *Cylindrotheca* cf. *closterium*) to 50.85% (*Biddulphia* cf. *aurita* +*Schizothrix* cf. *calcicola*). The EAA/NEAA ratios ranged from 0.97 to 2.12 for the same dual microalgal biofilms mentioned above (Table 3a, b). Significantly positive Pearson's correlations were observed between larval attachment and TAA, and between metamorphosis and TAA only (Table 3.4).

3.5 Discussion

The results from these experiments indicate that mixed microalgal biofilms have a positive effect on abalone (*H. iris*) attachment, metamorphosis, settlement, and survival compared to surfaces without biofilms (control plates). The results also suggest that some combinations of microalgal species better enhance these larval processes than others. While

no clear patterns were observed between the amount of individual amino acids within the biofilms and the larval responses, the total amino acid (TAA) correlated well with percent attachment and percent metamorphosis. Unfortunately, amino acid profiles for dual microalgal biofilms after 21 days were not available to determine correlations with percent settlement and survival (14 days larval exposure to a 7-day old biofilm). Since biofilms are likely to change in their amino acid profiles over time, it was not possible to use the 14-day old biofilm profiles for settlement and survival comparisons. Regardless, higher amounts of amino acids have been associated with more extra-cellular polymers (ECPs) within the biofilm matrix (Lam et al., 2005). Thus, it is likely that *H. iris* larvae are attracted for initial attachment and are induced to metamorphose by better established biofilms (greater quantities of ECPs) compared to young biofilms (less quantities of ECPs) or clean surfaces (no biofilms). Indeed Roberts et al. (2007) found that older single microalgal species biofilms induced *H. iris* attachment, metamorphosis, settlement, and survival compared to equivalent young biofilms. ECPs by themselves also have been found to induce settlement in marine invertebrates (Lam et al., 2005; Chen, 2007). Based on ECP extractions from twelve benthic diatoms, Chen (2007) found that a greater amount of ECP resulted in higher larval settlement for the abalone *H. diversicolor*.

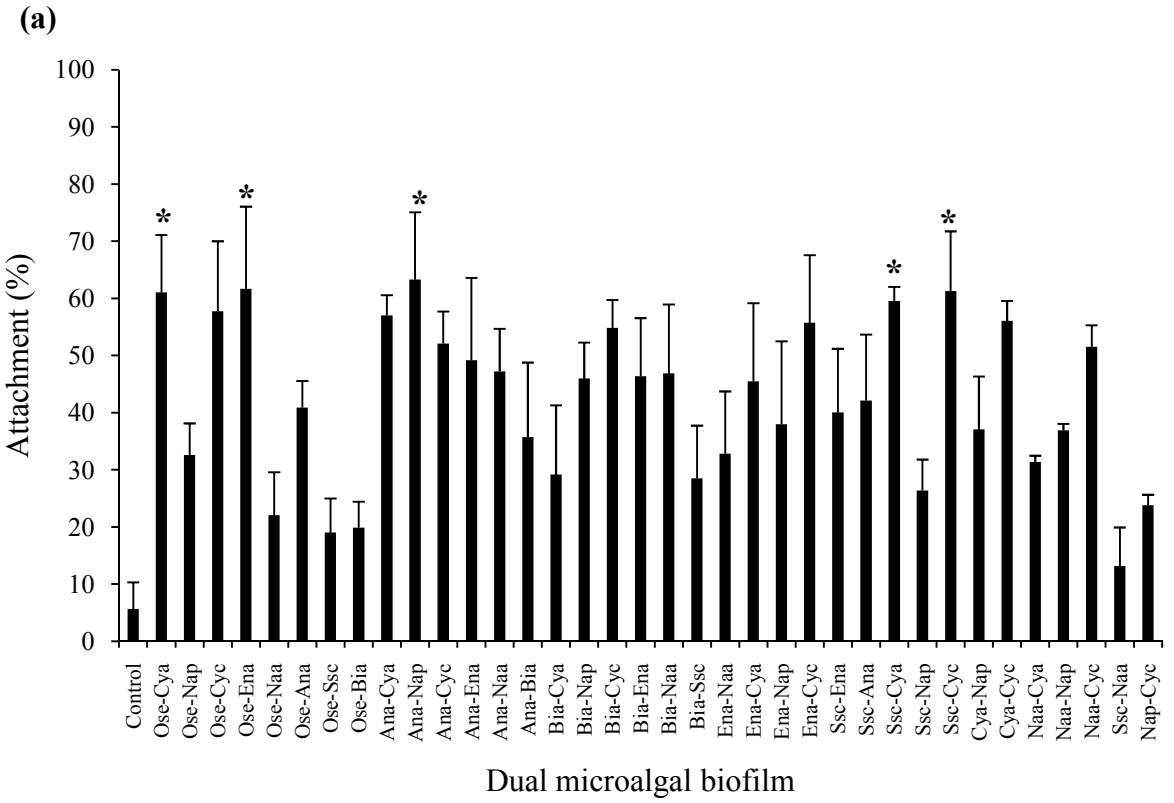
While dual microalgal biofilms appeared to induce a relatively high percent initial attachment (up to 65%), percent metamorphosis after 7 days and percent settlement and survival after 14 days were low (less than 35, 10 and 30%, respectively). These results may be due to a variety of reasons, including insufficient biofilm (and chemical cue) within the Petri plates, or a limited nutritional value from the combination of only two microalgal species within the biofilm. Indeed, Krug and Zimmer (2000) found that a waterborne compound extracted from the red alga, *Vaucheria longicaulis*, induced settlement in gastropod (*Alderia modesta*) larvae at high concentrations (0.67 mg/mL), but did not induce metamorphosis at lower concentrations (0.14 mg/mL). In addition, Viana et al. (2007) tested the digestive enzyme activity (lipase) in *H. flugans* when fed different microalgal species and found that survival of post-larvae was higher with higher amounts of lipids in their diets compared to diets with low lipid contents.

Based on the significant morphological and physiological differences between larvae and post-larvae, it is likely that abalone post-larvae require different biofilm surfaces from pre-settlement larvae (non-feeding larvae), and once settled, the post-larvae will begin rapid

consumption of the biofilm. Thus, the low settlement and survival numbers observed in this study may be a result of insufficient food or nutrient contents to support post-larval development. Similar results also were obtained for *H. rubra* larvae, which were allowed to feed on diatoms of various nutritional values (Daume, 2003). Based on this information, it is likely that a more complex biofilm (i.e, containing higher diversity of organisms and/or more nutrients) than those provided in this study may be required for *H. iris* post-larval developmental processes. Indeed, Dahms et al. (2004) found that natural biofims, which contained a high diversity of microorganisms, resulted in higher bryozoan larval settlement than mono-specific (diatom or bacteria) biofilms. While biofilms containing two microalgal species were used in the present study, these mixtures may not have provided enough biofilm "complexity" compared to biofilms with a greater number of organisms.

The combination of species within the biofilm also may be of importance in supporting post-larval development. For example, the best-performing biofilms in this study almost always contained cyanobacteria (*Oscillatoria* cf. *erythrata*, *Anacystis* cf. *aeruginosa*, and *Schizothrix* cf. *calcicola*). These species are not commonly used in hatchery environments (Kawamura et al., 1998; Daume et al., 1999; Roberts, 2001a, b), but form part of the natural diets of abalone (Tahil and Juinino-Menez, 1999), and have been shown to improve larval processes in previous studies (Morse and Morse, 1984b; Tahil and Juinino-Menez, 1999; Nagarkar et al., 2004). For example, Morse and Morse (1984) used extracts of cyanobacteria, such as *Synechococcus* spp. to induce settlement of *H. rufescens* larvae, and Nagarkar et al. (2004) demonstrated that cyanobacteria have high protein contents and may increase the nutritional value of a biofilm. This information is in agreement with the findings of the present study, in that the total amino acid content of the biofilms containing cyanobacteria was generally higher than those lacking cyanobacteria. Future studies may focus on more in-depth analyses (e.g., fatty acids, quorum sensing) of natural biofilms containing cyanobacteria, and their effects on abalone larvae/post-larvae behavior.

Figure 3. 1. Percent (a) attachment (n=3) on day 7th, and (b) metamorphosis (n=3) on day 7th. Asterisks (*) denote significant differences from the control, resulting from Dunn's tests ($p < 0.05$).



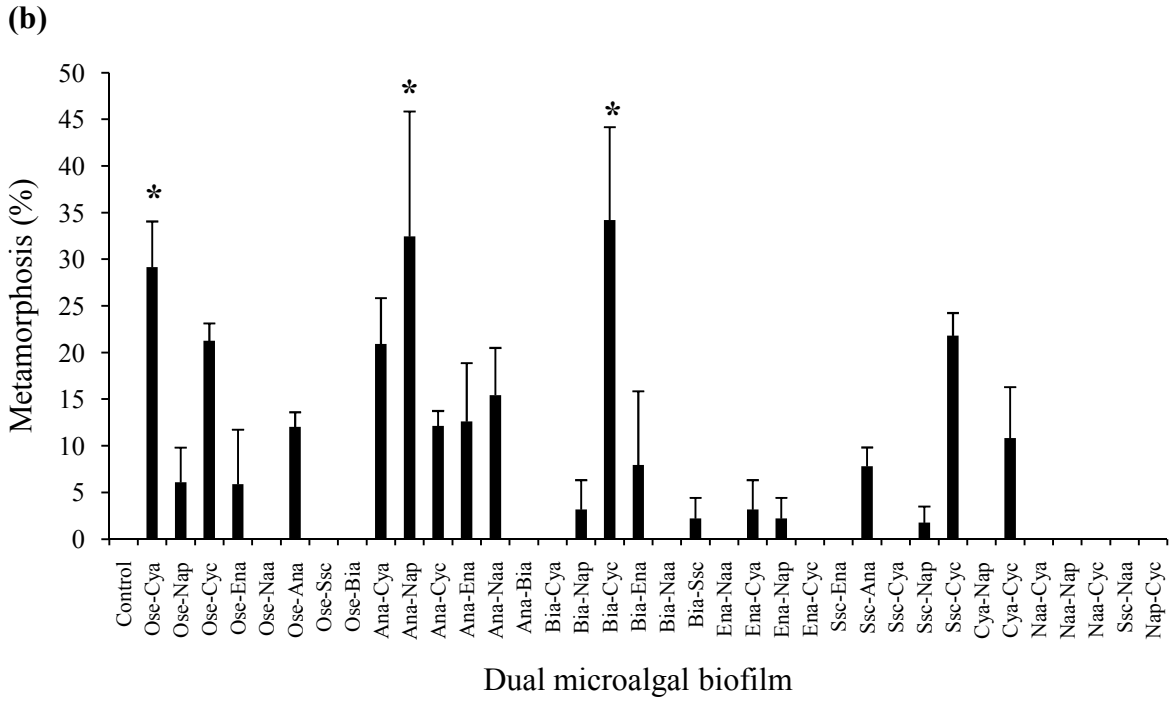


Figure 3. 2. Percent (a) settlement (n=3) on day 14th, and (b) survival (n=3) on day 14th. Asterisks (*) denote significant differences from the control, resulting from Dunn's tests ($p < 0.05$).

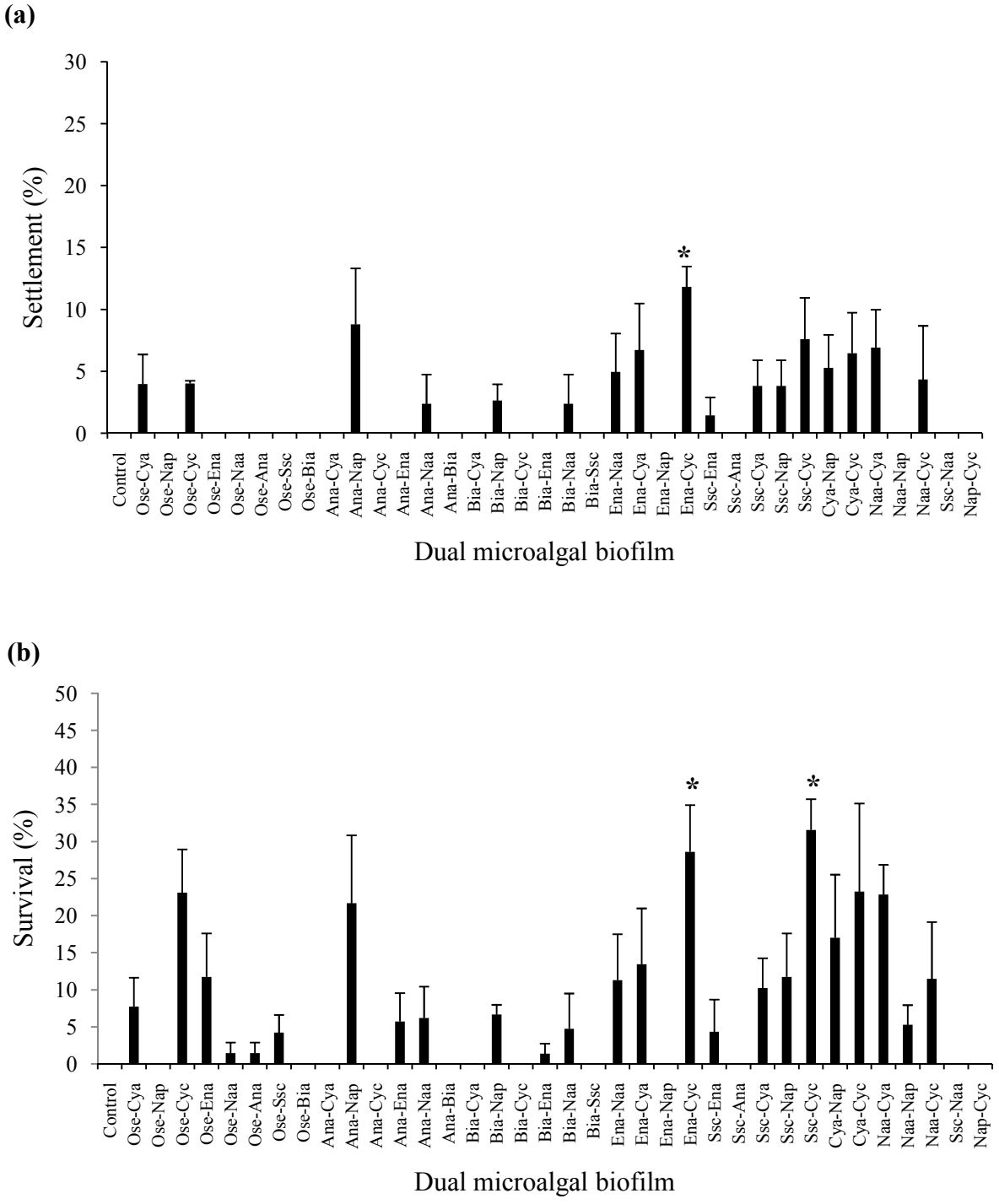


Table 3. 1. Kruskal-Wallis test on larval attachment and metamorphosis on day 7th, with dual microalgal biofilm as a fixed factor. Alphabetic letters denote significant differences resulting from Dunn's tests ($p < 0.05$), $n = 3$.

	Attachment			Metamorphosis		
Strain	Median	Rank	Z	Median	Rank	Z
Control	0.09	4.5	-2.81 [a]	0.00	34.5	-1.17 [a]
Ose-Cya	0.55	84.8	1.57 [b]	0.27	104.3	2.64 [b]
Ose-Nap	0.38	40.3	-0.85[ab]	0.05	63.5	0.41[ab]
Ose-Cyc	0.52	79.7	1.29[ab]	0.20	97.8	2.28[ab]
Ose-Ena	0.58	83.8	1.52[b]	0.00	53.3	-0.15[ab]
Ose-Naa	0.28	25.5	-1.66[ab]	0.00	34.5	-1.17 [a]
Ose-Ana	0.38	54.5	-0.08[ab]	0.12	83.2	1.48[ab]
Ose-Ssc	0.18	18.3	-2.05[ab]	0.00	34.5	-1.17 [a]
Ose-Bia	0.19	20.0	-1.96[ab]	0.00	34.5	-1.17 [a]
Ana-Cya	0.60	84.8	1.57[ab]	0.25	96.2	2.19[ab]
Ana-Nap	0.68	89.7	1.84 [b]	0.32	98.5	2.32 [b]
Ana-Cyc	0.53	76.7	1.13[ab]	0.32	98.5	2.32[ab]
Ana-Ena	0.50	67.2	0.61[ab]	0.11	83.7	1.51[ab]
Ana-Naa	0.44	67.0	0.60[ab]	0.19	74.8	1.03[ab]
Ana-Bia	0.32	46.8	-0.50[ab]	0.14	87.7	1.73 [a]
Bia-Cya	0.19	34.2	-1.19[ab]	0.00	34.5	-1.17 [a]
Bia-Nap	0.52	64.2	0.45[ab]	0.00	34.5	-1.17 [a]
Bia-Cyc	0.57	82.5	1.45[ab]	0.43	102.0	2.51 [b]
Bia-Ena	0.50	66.3	0.56[ab]	0.00	48.8	0.39[ab]
Bia-Naa	0.55	68.2	0.66[ab]	0.00	56.3	0.02 [a]
Bia-Ssc	0.22	32.7	-1.27[ab]	0.00	34.5	-1.17 [a]
Ena-Naa	0.26	40.0	-0.87[ab]	0.00	47.3	-0.47 [a]
Ena-Cya	0.40	62.0	0.33[ab]	0.00	34.5	-1.17 [a]
Ena-Nap	0.27	51.0	-0.27[ab]	0.00	48.8	-0.39 [a]
Ena-Cyc	0.48	76.0	1.09[ab]	0.00	47.3	-0.47 [a]
Ssc-Ena	0.45	54.7	-0.07[ab]	0.00	34.5	-1.17 [a]
Ssc-Ana	0.35	58.3	0.13[ab]	0.00	75.3	1.05 [a]
Ssc-Cya	0.58	91.2	1.92 [b]	0.07	34.5	-1.17 [a]
Ssc-Nap	0.24	29.2	-1.46[ab]	0.00	46.3	-0.53 [a]
Ssc-Cyc	0.60	86.2	1.65 [b]	0.00	98.7	2.33[ab]
Cya-Nap	0.42	48.8	-0.39[ab]	0.20	34.5	-1.17 [a]
Cya-Cyc	0.56	83.8	1.52[ab]	0.00	71.5	0.85[ab]
Naa-Cya	0.31	38.7	-0.95[ab]	0.15	34.5	-1.17 [a]
Naa-Nap	0.37	48.2	-0.43[ab]	0.00	34.5	-1.17 [a]
Naa-Cyc	0.55	75.7	1.07[ab]	0.00	34.5	-1.17 [a]
Ssc-Naa	0.07	11.5	-2.43[ab]	0.00	34.5	-1.17 [a]
Nap-Cyc	0.23	25.5	-1.68[ab]	0.00	34.5	-1.17 [a]
Overall		56.0			56.0	
	H = 62.49 df = 36 p = 0.004 (adjusted for ties)			H = 87.19 df = 36 p = 0.001 (adjusted for ties)		

Table 3. 2. Kruskal-Wallis test on larval survival and settlement on day 14th, with dual microalgal biofilm as a fixed factor. Letters denote significant differences resulting from Dunn's tests ($p < 0.05$), n = 3.

	Settlement			Survival		
Strain	Median	Rank	Z	Median	Rank	Z
Control	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Ose-Cya	0.04	72.0	0.87[ab]	0.11	62.8	0.37[ab]
Ose-Nap	0.00	38.50	-0.95 [a]	0.00	31.0	-1.36 [a]
Ose-Cyc	0.04	83.5	1.50[ab]	0.18	95.7	2.16[ab]
Ose-Ena	0.00	38.5	-0.95 [a]	0.17	71.3	0.84[ab]
Ose-Naa	0.00	38.5	-0.95 [a]	0.00	43.2	-0.70 [a]
Ose-Ana	0.00	38.5	-0.95 [a]	0.00	43.2	-0.70 [a]
Ose-Ssc	0.00	38.5	-0.95 [a]	0.04	57.2	0.06[ab]
Ose-Bia	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Ana-Cya	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Ana-Nap	0.04	94.3	2.09[ab]	0.17	90.7	1.89[ab]
Ana-Cyc	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Ana-Ena	0.00	38.5	-0.95 [a]	0.04	58.7	0.15[ab]
Ana-Naa	0.00	57.5	0.08[ab]	0.04	60.8	0.26[ab]
Ana-Bia	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Bia-Cya	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Bia-Nap	0.04	68.7	0.69[ab]	0.07	70.0	0.76[ab]
Bia-Cyc	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Bia-Ena	0.00	38.5	-0.95 [a]	0.00	41.8	-0.77 [a]
Bia-Naa	0.00	57.5	0.08[ab]	0.00	48.7	-0.40[ab]
Bia-Ssc	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Ena-Naa	0.04	75.0	1.04[ab]	0.13	69.0	0.71[ab]
Ena-Cya	0.07	80.3	1.33[ab]	0.14	72.0	0.87[ab]
Ena-Nap	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Ena-Cyc	0.13	105.2	2.68 [b]	0.29	100.7	2.44 [b]
Ssc-Ena	0.00	55.3	-0.04[ab]	0.00	47.8	-0.45[ab]
Ssc-Ana	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Ssc-Cya	0.04	74.3	1.00[ab]	0.09	77.7	1.18[ab]
Ssc-Nap	0.04	74.3	1.00[ab]	0.17	71.3	0.84[ab]
Ssc-Cyc	0.04	93.8	2.06[ab]	0.30	104.3	2.64 [b]
Cya-Nap	0.07	78.3	1.22[ab]	0.25	76.8	1.14[ab]
Cya-Cyc	0.09	81.0	1.36[ab]	0.30	81.8	1.41[ab]
Naa-Cya	0.04	89.5	1.83[ab]	0.21	96.0	2.18[ab]
Naa-Nap	0.00	38.5	-0.95 [a]	0.07	59.2	0.17[ab]
Naa-Cyc	0.00	61.3	0.29[ab]	0.08	68.3	0.67[ab]
Ssc-Naa	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Nap-Cyc	0.00	38.5	-0.95 [a]	0.00	31.0	-1.36 [a]
Overall		56.0			56.0	
	H = 62.49 df = 36 p = 0.004 (adjusted for ties)			H = 70.51 df = 36 p = 0.001 (adjusted for ties)		

Table 3. 3a. Amino acid profiles for dual microalgal biofilms (14 days old). The amount of each amino acid is presented as the mean of three replicates, with units of mg/100mg. Total amino acids (TAA) are shown as μg /cell. Percent essential amino acids (EAA), and non-essential amino acids (NEAA) also are shown.

Strain A	Ose								Ana						Bia			
Strain B	Cya	Nap	Cyc	Ena	Naa	Ana	Ssc	Bia	Cya	Nap	Cyc	Ena	Naa	Bia	Cya	Nap	Cyc	Ena
Asx	5.40	6.36	4.61	7.01	5.84	6.25	5.37	6.46	7.05	5.23	6.83	6.39	7.39	9.79	3.30	7.84	6.74	8.59
Glx	10.21	10.56	9.63	10.80	10.04	10.48	10.88	10.71	9.56	8.74	9.51	12.86	11.70	11.10	4.21	10.84	7.45	15.28
Ser	15.76	14.50	14.52	13.63	15.28	15.93	16.34	16.36	15.59	14.16	15.69	13.51	14.80	16.67	13.02	14.00	15.00	19.06
His	2.69	2.50	2.95	3.53	1.99	4.07	2.80	2.30	2.72	2.64	2.64	4.35	3.16	4.02	3.64	2.58	2.69	2.59
Gly	6.71	6.80	8.85	3.06	6.29	7.58	7.84	5.32	5.40	11.06	5.44	2.93	3.81	3.61	4.94	5.16	6.69	1.76
Thr	9.42	9.78	8.98	9.93	9.87	9.40	8.91	9.57	10.08	8.92	10.24	9.37	9.85	9.84	13.02	9.55	9.31	9.74
Ala + Tau	15.10	10.92	14.38	15.05	10.46	11.13	10.56	10.07	13.75	12.26	11.43	16.17	12.07	7.74	15.27	12.75	15.58	4.86
Arg	2.67	2.56	2.55	2.63	2.85	2.98	2.91	2.71	2.65	2.68	2.60	2.49	2.58	2.67	2.74	2.32	2.65	1.86
GABA	0.05	0.06	0.06	0.03	0.07	0.10	0.12	0.06	0.05	0.09	0.04	0.04	0.10	0.01	0.13	0.14	0.05	0.08
Tyr	2.31	2.82	2.22	2.78	2.72	2.60	2.69	2.80	2.54	2.51	2.60	0.26	2.60	2.61	3.96	2.60	2.45	2.53
Met	0.33	1.63	0.71	1.79	0.87	0.42	1.00	1.45	0.55	0.60	1.44	0.39	1.27	0.66	1.63	0.86	1.98	1.01
Val	3.86	4.96	3.70	5.32	4.60	1.23	3.49	4.76	4.46	4.03	4.44	5.83	4.81	5.35	5.75	4.24	4.04	6.02
Phe	6.53	7.87	6.39	7.63	7.57	7.19	6.53	7.27	7.46	7.07	7.74	8.20	8.06	8.30	10.18	8.54	7.37	7.23
Ile	3.11	4.59	2.96	3.94	4.10	3.93	3.23	3.30	3.41	3.99	3.49	4.16	3.94	4.54	4.68	3.58	3.23	3.57
Leu	8.86	10.76	8.23	10.29	10.84	9.16	9.80	10.08	9.73	10.01	10.04	11.07	10.86	11.13	8.71	10.02	9.21	9.83
Orn	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.02
Lys	6.99	3.32	9.24	2.54	6.62	7.55	7.53	6.76	4.99	6.00	5.81	1.95	2.99	1.94	4.83	4.99	5.58	5.98
Pro	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TAA	0.30	0.08	0.27	0.22	0.17	0.18	0.16	0.11	0.25	0.30	0.29	0.24	0.22	0.24	0.21	0.12	0.20	0.21
EAA (%)	56.82	56.25	57.54	60.10	56.86	54.13	53.97	55.36	57.12	55.61	57.24	61.50	57.02	53.47	67.74	57.26	59.04	50.00
NEAA (%)	43.18	43.75	42.46	39.90	43.14	45.87	46.03	44.64	42.88	44.39	42.76	38.50	42.98	46.53	32.26	42.74	40.96	50.00
EAA/NEAA	1.32	1.29	1.35	1.51	1.32	1.18	1.17	1.24	1.33	1.25	1.34	1.60	1.33	1.15	2.10	1.34	1.44	1.00

Table 3.3b. Amino acid profiles for dual microalgal biofilms (14 days old). The amount of each amino acid is presented as the mean of three replicates, with units of mg/100mg. Total amino acids (TAA) are shown as μg /cell. Percent essential amino acids (EAA), and non-essential amino acids (NEAA) also are shown.

Strain A	Bia		Ena				Ssc						Cya		Naa			Nap
Strain B	Naa	Ssc	Naa	Cya	Nap	Cyc	Ena	Ana	Cya	Nap	Cyc	Naa	Nap	Cyc	Cya	Nap	Cyc	Cyc
Asx	8.59	7.13	5.77	5.79	5.78	6.90	4.93	7.20	7.02	4.63	5.78	7.12	5.58	5.79	5.83	5.14	6.43	3.61
Glx	10.37	8.04	11.86	9.43	9.51	11.50	9.32	9.37	10.18	8.28	11.16	10.94	5.91	6.36	9.72	9.08	9.68	4.18
Ser	14.96	24.35	13.47	14.90	15.17	15.10	14.22	14.55	15.59	13.78	15.60	15.82	14.33	14.49	15.07	14.09	14.30	12.92
His	2.23	3.39	2.72	2.23	2.48	2.34	2.65	4.71	2.22	4.15	2.10	2.96	2.90	2.84	2.69	3.78	2.53	2.86
Gly	2.02	6.57	4.34	4.16	8.52	3.82	6.07	5.05	4.46	16.90	4.94	3.81	6.39	6.36	6.03	12.93	4.52	4.79
Thr	10.10	9.46	8.83	9.84	9.35	10.38	8.76	9.15	9.94	7.73	9.80	10.15	11.16	10.22	9.41	8.31	9.52	13.75
Ala + Tau	8.83	6.17	16.11	9.92	11.47	14.22	12.22	9.07	16.64	13.01	16.71	11.44	15.34	16.03	13.35	11.97	19.38	15.97
Arg	2.49	2.39	2.48	2.67	2.43	2.82	2.65	2.68	2.61	2.30	2.49	2.51	2.90	2.78	2.65	2.46	2.86	2.73
GABA	0.05	0.09	0.04	0.07	0.09	0.04	0.09	0.10	0.09	0.01	0.04	0.06	0.05	0.05	0.16	0.15	0.05	0.12
Tyr	2.71	2.34	2.49	2.70	2.64	2.50	2.61	2.38	2.37	2.14	2.28	2.69	3.07	2.74	2.50	2.48	2.29	3.68
Met	0.47	0.97	0.40	0.39	0.90	0.27	1.13	1.03	0.92	0.62	1.40	0.85	1.35	1.70	0.72	0.75	1.60	2.47
Val	5.89	4.49	4.57	5.27	4.63	4.79	4.44	4.20	4.16	2.68	3.77	5.64	4.65	4.26	4.81	3.67	4.16	5.64
Phe	9.86	6.98	7.94	9.87	7.74	7.90	8.79	8.28	7.01	5.32	6.99	8.11	8.21	7.88	7.63	6.56	6.68	9.64
Ile	5.50	3.47	3.87	5.26	5.09	3.86	4.12	4.18	3.36	2.74	3.29	4.16	3.52	3.31	3.52	3.15	3.21	4.18
Leu	12.99	9.27	10.45	12.90	9.68	10.26	11.28	11.73	9.11	8.30	9.36	11.15	8.99	9.17	9.78	7.99	8.34	8.20
Orn	0.01	0.02	0.00	0.01	0.02	0.00	0.01	0.02	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.00
Lys	2.95	4.87	4.66	4.58	4.50	3.28	6.70	6.30	4.30	7.40	4.28	2.57	5.66	6.01	6.13	7.50	4.44	5.24
Pro	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TAA	0.18	0.21	0.10	0.28	0.15	0.23	0.24	0.24	0.29	0.18	0.27	0.17	0.14	0.27	0.18	0.07	0.16	0.20
EAA (%)	58.78	49.15	60.29	59.62	55.77	57.35	60.07	58.49	57.66	51.87	57.72	57.06	61.74	61.37	58.08	53.44	59.87	67.96
NEAA (%)	41.22	50.85	39.71	40.38	44.23	42.65	39.93	41.51	42.34	48.13	42.28	42.94	38.26	38.63	41.92	46.56	40.13	32.04
EAA/NEAA	1.43	0.97	1.47	1.52	1.26	1.34	1.51	1.41	1.36	1.08	1.37	1.33	1.61	1.59	1.39	1.15	1.49	2.12

Table 3. 4. Pearson's correlations between larval parameters (percent attachment, metamorphosis, settlement and survival) and biofilm amino acid contents (TAA = total amino acids, EAA = essential amino acids, NEAA = non-essential amino acids, and EAA/NEAA ratios). Bold numbers denote significant differences ($p < 0.05$). Correlation coefficients (R) and p-values (p) are shown. Dashes (-) indicate values not measured.

	Attachment		*Metamorphosis*		*Settlement*		*Survival*	
	R	p	R	p	R	p	R	p
TAA	0.453	**0.001**	0.447	**0.001**	-	-	-	-
EAA	0.054	0.580	-0.053	0.580	-	-	-	-
NEAA	-0.054	0.580	0.053	0.580	-	-	-	-
EAA/NEAA Ratio	0.001	0.998	-0.087	0.371	-	-	-	-

Chapter 4 Alternative protein sources in artificial feeds for New Zealand Black-footed abalone (*Haliotis iris)* juveniles

4.1 Abstract

Growth and health parameters were tested in juvenile New Zealand black-footed abalone (*Haliotis iris*) fed nine diets containing different protein sources (white and red fish meal, blood meal, meat and bone meal, casein, soybean concentrate, wheat gluten, maize gluten and *Spirulina* powder) over a five-month period. The growth parameters measured included maximum shell length, total animal weight, and soft body (including gonad) and gonad weights. The health indicators included survival, goblet cell counts and epithelium thickness of the tentacle region. Proximate analyses and amino acid and fatty acid profiles also were determined on the diets, soft body (including gonad) and shell materials to evaluate the overall nutrient contents (diet and animal) and requirements (animal). Measurements of soft body (including gonad), gonad, and shell growth parameters indicated that white and red fish meals generally result in better growth performance, although not statistically different from the next-best diets, which were casein, soybean and *Spirulina*. However, these diets were significantly better than blood meal, meat and bone meal, wheat gluten and maize protein diets, which were not suitable for abalone juvenile growth. Animals fed blood meal had the lowest survival rates and crude protein contents. *Spirulina* produced animals with the heaviest shells and soybean treatments produced animals with the highest gonad to soft body (excluding gonad) ratios. Significant differences were observed in the soft body (including gonad) amino acid profiles of animals across treatments. GABA, albeit absent in both soft-bodies and diets, accounted for 1-5% of the total amino acids in both newly-grown and old shells. Significantly lower acid/basic amino acid ratios were found in fish meal diets, indicating that animals fed fish protein may incorporate more aragonite than calcite in their shells. Low methionine contents (deficiency) in some diets, such as in soybean diets, appeared to not affect the growth of *H. iris* juveniles, suggesting that the methionine requirements in this abalone species are low. The commercial implications of this study are that, while a fish meal diet will achieve good growth and health in *H. iris*, companies wanting to lower feed costs may entirely or partially substitute this protein source with soybean meal or *Spirulina* protein without any significant changes to the performance of juveniles of this abalone species.

4.2 Introduction

Commercially produced artificial diets for abalone aquaculture have been used since the late 1990s. However, research on these diets has mainly been focused on supplementing or replacing seaweeds (Uki, et al., 1986a; Uki and Watanabe, 1992; Britz, 1996 a, b; Boarder and Shpigel, 2001; Serviere-Zaragoza et al., 2001). Advantages of using these commercial feeds include easy storage and cleaning, and less feeding labour costs. In addition, many of these products result in better animal growth, compared to macroalgae (Hahn, 1989b, Fleming, 1996). For example, Viana et al. (1993) observed nearly four times faster growth rates in *H. fulgens* juveniles fed artificial feeds made of processed ingredients (fish meal and casein) than those fed macroalgae. One of the main differences between formulated feeds and seaweed diets is that macroalgae generally contain about 7-15% protein (Emmanuel and Corre, 1996), and less lipids than carbohydrates (Uki et al., 1986a). Conversely, artificial diets can be formulated to provide abalone with more balanced nutrients and an overall greater amount of protein and lipids to boost growth. However, optimizing and balancing the components within artificial diets, especially protein, necessitates specific research on the requirements of each abalone species and their culturing conditions.

In abalone aquaculture, casein and fish meal are the most commonly used protein sources in artificial feeds. These protein sources are highly effective at promoting growth, although casein is more expensive than most protein types. Other protein types used in abalone feed formulation include poultry and cattle by-products, plant material, and microalgal protein (Thongrod et al., 2003; Stott et al., 2004). The amount and combinations of different types of protein are hugely variable, and so are their effects on different abalone species (Uki et al., 1985; Flemming et al., 1996; Bautista-Teruel et al., 2003). Thus, the optimization of feed formulation for a particular abalone species requires an in-depth analysis of each individual protein source and how different combinations may improve growth. For example, fish meal is a good stand-alone protein source for many abalone species, such as *H. discus hannai* (Ogino and Kato, 1964) and *H. midae* (Britz, 1996b). However, cattle blood protein often is used as an additional protein source in other species, such as *H. rubra* (Flemming et al., 1996), but by itself does not provide sufficient nutrients for growth. Plant protein from field peas, faba beans, yellow lupins, defatted soyflour, and vetch and ray grass concentrates also have been incorporated in formulated feeds, and tested with a wide range of results (Uki et al., 1985; Vandepeer et al., 1999). The specific requirements of each species may revolve around the need to consume particular amino acids, such as methionine and taurine (Gaylord et al.,

2007). To meet nutrient balance, mixed protein sources are commonly used, such as plant protein, casein, fish meal and gelatine. For example, one of the best protein combinations has been shown to be fish meal (7.5%), shrimp meal (7.5%), and soybean meal (35%), which was shown to result in weight gains of over 450% in *H. asinina* (Bautista-Teruel et al., 2003). These types of combinations not only produce better growth results, but may reduce feed costs substantially.

The New Zealand black-footed abalone, *H. iris*, has been developing as an aquaculture species since the 1990s (Tong and Moss, 1992). However, early cultivation was created around drift seaweed diets, such as *Ulva lactuca, Gracilaria chilensis,*and *Macrocystis pyrifera* (Stuart and Brown, 1994). The next developments occurred in 1993 when a New Zealand dairy company (Promak Technology Ltd.) began to produce commercial pellets, which contained 42% casein as the main protein source (Flemming et al., 1996). Unfortunately, this company stopped production prior to 2000, and no further research on the nutrient requirements of this species has taken place. Currently, the most commonly used commercial products are AbFeed™ pellets (Sea Plant Products, South Africa) and Adam and Amos abalone food (Australia). However, these feeds have not been specifically designed for *H. iris*, and may not necessarily be optimal for growth of this species. In addition, the high product and importing costs make these foreign diets prohibitive for small companies, which still revert to natural seaweeds. Thus, using cheap local materials (i.e., meat and bone meal, soybean) to create a feed to the specific requirements of *H. iris* may be greatly beneficial for the New Zealand abalone industry. In order to advance in this commercial area, a clear understanding of the basic nutrient requirements of this species is needed. The aims of this study were to test nine different protein sources, including fish (white and red fish meals), poultry products (blood meal, meat and bone meal, and casein), plant material (soybean concentrate, wheat gluten, and maize), and microalgae (*Spirulina*) for individual formulated feeds on juvenile *H. iris* over a five-month period. Growth performances and health conditions were evaluated in soft body (including gonad), gonad, and shell materials through proximate analysis, and amino acid and fatty acid profiles to identify the best protein source for this species.

4.3 Materials and Methods

4.3.1 Experimental animals and culturing system

Cultured *H. iris* juveniles were produced at Seahorses Australasia Limited, Warrington, Dunedin, New Zealand (45°42'21.64"S, 170°36'9.15"E). The animals were about 1.5 to 2 years old, and their sizes ranged between 20 and 22 mm in maximum shell length. All individuals were healthy and had clean shells at the start of the experiments.

Growth experiments were performed at Seahorses Australasia Limited. The juveniles were first acclimatized in a flow-through tank for one month before the experiments were started. During the acclimation period, the animals were fed a commercial diet (Adam and Amos abalone food, Australia). After the one-month period, the animals were carefully removed from the holding tank with a flat, blunt blade. Then, the animals were dried with paper towels, and their initial maximum shell lengths (mm) and total animal weights (g) were recorded. Lengths were measured with vernier calipers (Mercer 0-125 mm) to the nearest 0.1 mm, and weights were measured with a digital balance (Mettler Toledo AB204-S) to the nearest 0.1 g. Twenty randomly selected animals were placed in each of 27 experimental containers (three replicates for each of the nine diets). The containers (1 L in volume with square PVC lids, semi-transparent in color) had semi-recirculated (50% water exchange per day) filtered (10 μm filter) seawater. The temperature was maintained at 18±0.3 °C throughout the experimental period. The animals were fed one of nine diets, following a complete randomized design, for a period of five months. The daily feeding rate was 5% of the total body weight per tank. During this growth period, water flowed through the containers at a rate of 500 ml/hour, which equates with a total water exchange of 12 times per day. Water quality was always kept in good condition (pH between 8.3 to 8.5, dissolved oxygen > 10 ppm). Faeces and uneaten food residues were swept away every other day. An additional 24 animals were preserved (freeze dried) for amino acid and fatty acid analyses, and 3 animals were preserved in formalin for histological analyses (see below). These samples served as controls.

4.3.2 Experimental diets

Nine experimental diets were specifically formulated to contain about 33% protein and 5% crude lipids, while trying to maintain similarity in all other components (Table 4.1). All dried ingredients, except starch and cellulose, were ground up to 200 μm particle size, and then mixed in a blender. Starch was mixed with boiling water in order to activate the binding propensities (gelatinize). Then, the activated starch and cellulose were added to the mixture of dried ingredients. The lipid oil was then added to form a dough. The dough was spread on a flat, square-sized board to 3 mm thickness. Small pieces (1 cm^2) were cut with a knife just prior to drying in a Mcgreger hot-air oven at 45 °C for about 12 hours, at which point the moisture level was reduced to less than 15%. The diets were then stored in a -20 °C freezer until they were used. Proximate analyses, amino acid and fatty acid analyses of these diets also were performed as indicated below.

4.3.3 Growth and health measurements

At the end of the five-month experiment, the maximum shell length and total animal weight of each individual were carefully measured, and survival rates were recorded. Three of the 20 animals per container were used to obtain a histological section of the tentacle region, from which goblet cell counts and epithelium thickness measurements were made (Fig. 4.1). The remaining animals per container (depending on mortality) were dissected to separate the shell, gonad and soft body (excluding gonad). Wet and dry weights and moisture contents were obtained for each of these three components. The pooled dried gonad and soft tissues of the individuals within each container were ground up for proximate analyses and amino acid profiles.

4.3.4 Histological analysis

The animals selected for histological sections (see above) were fixed in 10% buffered formalin for 48 hr. Then, the animals were embedded in paraffin, and two 4 μm sections of the front tentacle area were cut with a rotary microtome (Leica RM2235). One of these sections was stained with Haematoxylin and Eosin to highlight the epithelial layer, and the

other section was stained with Periodic Acid-Schiff (PAS) to highlight the tentacle mucus cells. Photographs of all sections were obtained under an Olympus BX41 light microscope. Within these thin sections, three randomly chosen 50 μm-length cross-sections of epithelial cell regions were used to count all goblet cells and to measure the epithelial thickness. The epithelium layer thickness was determined to be the longest length of epithelium which included the apical border. All measurements were performed with the NIH J program (provided by the Research Service Branch of the National Institute of Mental Health, NIH).

4.3.5 Proximate analyses

Proximate analyses were conducted on diet and soft tissue samples to obtain crude protein, crude lipid, ash and moisture contents following AOAC (1995). The Kjeldahl method was used for crude protein determination and the Bligh and Dyer (1959) method for crude lipid determination.

For crude protein extractions, about 0.5-1 g of dried diet and 0.2-0.3 g of dried abalone samples were used. These samples were digested in Velp tubes with 3 g of catalyst (9:1 mixture of potassium sulfate and cupric sulfate) and 10 ml of concentrated sulfuric acid. The tubes with samples and blanks (no sample material) were placed in a Velp digester (Velp Scientifica Ltd.) within a hood. All samples were boiled for 3 hrs, and then diluted to 100 ml with mili-Q water. Five ml of each diluted digestion sample were distilled in a UDK126 semi-automatic steam distilling unit (Velp Scientifica Ltd.). Then, the ammonia in these samples was collected in 250 ml Erlenmeyer glass flasks with 10 ml of 0.05 N sulfuric acid and two drops of indicator, until a total volume of 150 ml was obtained. The indicator solution was made up by mixing 100 ml of 0.2% methyl red solution and 50 ml 0.1% methylene blue in ethanol. The amount of ammonia in each sample was obtained by titrating with standardized 0.05 N sodium hydroxide solution. The percent of crude protein was calculated with the formula:

$$\text{Crude protein (\%)} = 0.0007 \times (Vb - Vs) \times (F \times 6.25 \times 20 / S) \times 100$$

Where, Vb = volume of standard 0.05N sodium hydroxide solution required for the blank (ml), Vs = volume of standard 0.05N sodium hydroxide solution in the sample, F = correction factor for the standard 0.05N sodium hydroxide solution in this study (0.4185), and S = sample weight (g).

The lipid extract method was modified for small samples from Bligh and Dyer (1959). Dried diet (0.3 g) and abalone soft body (including gonad) (0.5 g) samples were hydrated in 0.7 ml distilled Milipore water and a 3ml mixture of methanol (2 ml) and chloroform (1 ml) in pre-weighed 25 ml screw top glass tubes. An ultrasonicator and nitrogen gas were used to enhance the extraction processes and to prevent oxidation. While ultrasonicating, 1 ml chloroform was added. After a 10 min interval, 1 ml of water was added, under nitrogen. The solution was then filtered with No.1 filter paper, and then centrifuged for 5 min at 2000 rpm. The lower organic solvent layer was collected into a pre-weighted test tube with a nitrogen stream gently blowing under a warm water bath until all the solvent was vaporized. Finally, the net weight of the lipids in the sample was recorded.

The amount of moisture in each diet and soft body (including gonad) sample was obtained from the net percent of water loss after freeze drying (Chist alpha series freeze dryer). The ash content was obtained by complete combustion in a 550 °C oven for 6 hrs. The mean protein gain per abalone was calculated with the following equation:

$$MPG\ (mg/abalone) = (SBt \times 1\text{-}Mt \times Pt) - (SBi \times 1\text{-}Mi \times Pi)$$ Mai et al. (1995b)

Where, SBt = final soft body (including gonad) weight (mg), Mt = moisture content (%), Pt = final protein content (%), SBi = initial soft body (including gonad) weight (mg), Mi = initial moisture content (%), and Pi = initial protein content (%).

4.3.6 Amino acid analyses

The amino acid analyses were conducted in accordance with Paramás et al. (2006). Three replicate 0.2 g dried powdered samples of abalone soft body (including gonad) and diets were each mixed with 5 ml of water in 25 ml screw top glass tubes. To each sample, a 5 ml 13 M

concentrate hydrochloric with 0.1% phenol solution was added, and heated in a Velp digester at 110 °C for 12 hrs under nitrogen gas. After cooling, the solution was adjusted to a pH of 6.5-7.5 by adding 1 M NaOH solution. The final volume was recorded and stored in a -20 °C freezer for further analysis. Samples were processed on a Shimadzu LC-10AD automatic sampling high-pressure liquid chromatography system (HPLC) fitted with a DGU-2A de-gas system with a helium gas tank.

A mixed L-amino acid standard was prepared in sodium phosphate buffer (10 mM, pH 7.3; BDH). This standard contained leucine (Leu) 1.844 g/L, isoleucine (Ile) 0.879 g/L, methionine (Met) 0.577 g/L, taurine (Tau) 0.189 g/L, tyrosine (Tyr) 0.125 g/L, glycine (Gly) 0.236 g/L, valine (Val) 0.925 g/L and proline (Pro) from BDH; aspartic acid (Asp) 0.059 g/L, alanine (Ala) 0.007 g/L, and phenylalanine (Phe) 0.354 g/L from Merck; and γ-aminobutyric acid (GABA) 1.650 g/L, ornithine (Orn) 0.930 g/L, histidine (His) 1.459 g/L, lysine (Lys) 0.361 g/L, glutamic acid (Glu) 0.272 g/L, threonine (Thr) 0.781 g/L, arginine (Arg) 0.381 g/L, serine (Ser) 0.395 g/L and 3,5-dibromotyrosine (I.S.) 1.785 g/L from Sigma.

In addition, an internal standard (3, 5-dibromotyrosine) was added to the samples and the mixed L-amino acid standard. This internal standard was added to provide a final concentration of $1x10^{-5}$ M. The samples were reacted with o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (MCE) at room temperature. The OPA regent was prepared by dissolving 500 mg of OPA (Merck) in 22.5 ml of ethanol. A total volume of 25 ml was obtained by adding 0.4 M borate buffer (pH 10) and 400 µl of 2-mercaptoethanol (Sigma). The OPA regent was stored in a dark screw-top bottle at 4 °C. To avoid photo-oxidation, a fresh reagent was prepared every three weeks. 50 µl of each sample was mixed with 450 µl sodium phosphate buffer (100 mM, pH 7.3; BDH) and 100 µl OPA regent and 50 µl of internal standard in a new 1.5 ml vial. These mixtures were prepared just prior to analyzing with HPLC. The reactions of the samples with OPA reagents yielded isoindolic derivatives to provide a fluorescence of analytes.

HPLC analyses were conducted with a solid phase consisting of a NOVA-PAK® C_{18} column (4 µm 3.9 × 150 mm Waters). The mobile phase was adapted from Paramás (2006) to provide a gradient elution. The first mobile phase was 80:19:1 ratio of sodium phosphate buffer (10 mM, pH 7.3)/methanol/tetrahydrofurane (solution A). The second mobile phase

contained a 20:80 sodium phosphate buffer (10 mM, pH 7.3)/methanol (solution B). Solution A was added at 100% at 0.1 ml/min for the first 3 min, after which time the flow rate was increased to 1.5 ml/min. At 14.5 min, the gradual addition of solution B was started. At 19.5 min, the ratio of solution A to B was 85:15. At 24.5 and 45 min, the ratios were 70:30 and 30:70, solution A to B, respectively. Fluorimetric detection of amino acids was obtained at excitation and emission wavelengths of 340 and 426 nm, respectively.

Because HPLC does not detect proline, visible spectroscopy was employed to the same sample extracts prior to pre-treatment with OPA to detect this amino acid according to Amerine and Ough (1980). 0.1 ml of the previously hydrolyzed sample was transferred to a 15 ml screw-top test tube. To this sample, 5 ml of pure water, 0.25 ml formic acid, and 1 ml methyl cellosolve (2- methoxyethenol) ninhydrine solution (3 g of ninhydrine dissolved into 100 ml 2- methoxyethenol) were added and boiled with the cap on for exactly 15 min in a water bath (Grant, SBB14). After cooling to room temperature, a 1ml isopropanol-water mixture (1:1 by volume) was added. These solutions were transferred into cuvettes to measure absorbance with a spectrophotometer (Ultrospec 21000pro UV/Visible) at a 517 nm wavelength. The results were calculated on the basis of the curve formed by the standard solution.

Shell protein extraction followed methods by Mai et al. (2003). Individual shells were kept in 5% NaOH for 7 hrs, then rinsed thoroughly with deionized water, and air-dried for 24 hrs. 1 g powdered samples of newly-formed green shell (NS) and old brown shell (OS) were obtained and suspended in 5 ml of deionized water. These samples were passed through 3500 molecular weight cut-off dialysis membranes (Serva 44311 membra-cel ® dialysis tubing MW cutoff 3500 22 mm) for 72 hrs at room temperature against an external solution of 5% acetic acid and 0.01% (w/v) sodium azide. Acetic acid was removed from the sample remaining in the dialysis tubing with ultra-pure water for 48 hrs, and centrifuged at 10,000 g for 30 min at 4 °C. The resulting shell matrix protein (SMP) was collected from the precipitate by re-suspending in 5 ml Milipore water for subsequent digestion and HPLC and proline analyses. The ratio of acidic to basic amino acids was calculated following Mai et al. (2003) as an index of the amount of calcite and aragonite in the shell. Specifically, the ratio was calculated as: (Asx + Glx) / (Lys + Arg + His).

4.3.7 Fatty acid composition

Purification of lipids

Sub-samples of 100 µl of dry lipid (from the crude lipid analysis) were re-suspended into 1 ml of chloroform. Then, 3 ml of 6% NaOH in methanol were added to each sample. The test tubes were sealed under nitrogen and placed in a boiling-water bath (Grant, SBB14) for exactly 20 minutes. Then, 5 ml pure water and 2 ml diethyl ether were added to the test tubes and mixed well. After settling (about 5 min), the upper organic layer was discarded. About 20 drops of concentrated hydrochloric acid (about 0.8 ml) and 2 ml of diethyl ether were added to each sample and shaken for 1 minute.

Methylation

The diethyl ether layer was transferred to a new test tube with 3 ml 14% Boron trifluoride in methanol and 0.1 ml of tricosanic acid (1 mg / ml internal standard). The test tubes were sealed under nitrogen flow, and heated for 20 min in boiling water.

Extraction of FAME

In order to extract the Fatty Acid Methyl Easters (FAMEs), the tubes were allowed to cool. Then, 5 ml of distilled water were added and shaken, followed by 2 ml of diethyl ether and further shaking. The samples were centrifuged for 3 min at 2000 rpm. The upper diethyl ether phases, which contained the FAMEs, were transferred into new test tubes. The samples were concentrated under nitrogen using a warm water bath to about 0.5-1 ml. The extracts were stored in nitrogen gas at -20°C until further analysis.

GC settings

The fatty acids were analyzed using a Shimadzu GC-17A GC-MS, containing a GC17A gas chromatography and Qp-5000 mass spectrometer. The carrier gas was purified helium with a 51.3 ml per minute flow rate (150 KPa in pressure). The splite mode was on, and the

ratio was 18. The temperature of the injector and interface was 250 °C. The GC column was a Varian Wcot fused silica capillary column CP-WAX 58 (FFAP-CB 25M×0.25 mm ID DF = 0.2). The mass spectrometer was set at 1.5 Kv detecting volts, and the mass range was between 40 to 350 with a 0.5 interval. The GC temperature program was set at 105 °C, lasting for 3 minutes and then increasing at a speed of 1 °C per minute until reaching 190 °C for 1 minute. The total running time was 91 minutes.

4.3.8 Statistical analyses

Analyses for all growth and health parameters, except for survival, were conducted with one-way ANOVAs (diet as fixed factor) with Tukey *post-hoc* tests. For survival analyses, a Kruskal-Wallis test was performed.

Individual amino acids and fatty acids within different abalone components also were analyzed with Kruskal-Wallis tests and Dunn's multiple comparisons. All analyses were performed using MINITAB version 14 software package.

4.4 Results

4.4.1 Growth parameters

Results from all shell and soft body (including gonad) growth parameters indicate that juveniles fed white fish meal, red fish meal, casein, soybean and *Spirulina* protein diets had similar growths, and these diets performed significantly better than blood meal, meat and bone meal, wheat gluten and maize protein (Figs. 4.2-3; Table 4.2). Shell growth measurements ranged from 3.50±0.21 mm with blood protein in the diet, to 8.79±0.40 mm with white fish protein in the diet. The mean shell growth (±SE) of the best-performing diet (white fish protein) was 1.76±0.08 mm per month. Shell weights ranged from 0.54±0.03 to 1.14±0.08 g (blood protein and white fish protein, respectively) for wet shell, and 0.49±0.03 to 1.05±0.03 g (blood protein and red fish protein, respectively) for dry shell. The wet weight gain of the entire animal (shell plus soft body [including gonad]) ranged from 0.33± 0.02 g for animals fed blood protein diets, to 1.79±0.07 g for animals fed diets with red fish protein. The mean animal weight gain (±SE) of the diets with best performance was 0.36±0.01 g per

month. Wet soft-body weights ranged from 0.54±0.04 to 1.32±0.07g (gluten protein and red fish protein, respectively). Dry soft-body weights ranged from 0.10±0.14 g with the blood meal diet and 0.24±0.01 g in the red fish diet. The mean soft-body weight gain (±SE) of the best-performing diet (red fish protein) was 0.15±0.01 g per month wet weight and 0.03±0.00 g per month dry weight.

Wet and dry soft-body to shell ratios (SB/S) resulted in significantly higher values for both white and red fish protein diets than others diets, whereas *Spirulina* protein diets resulted in significantly lower wet and dry weights (Fig. 4.4; Table 4.2). Wet SB/S ratios ranged from 1.15±0.06 with red fish protein to 0.85±0.06 with *Spirulina* protein. Dry SB/S ratios ranged from 0.23±0.00 with red fish protein to 0.17±0.01 with *Spirulina* protein.

Generally, gonad wet weights followed the same pattern as the growth parameters. White and red fish meals, soybean, and casein produced animals with significantly greater gonad wet weights (Fig. 4.5, Table 4.2). Gonad wet weights ranged from 0.06±0.00 g with maize protein to 0.16±0.01 g with soybean protein. Soybean diets also resulted in significantly higher gonad to soft body (excluding gonad) indices (GSI), while maize had the lowest GSI values. GSI measurements ranged from 0.11±0.01 with maize protein in the diets, to 0.17±0.01 with soybean protein in the diets.

4.4.2 Health and survival

Survival of juvenile *H. iris* fed different diets varied greatly throughout the five-month experimental periods (Fig. 4.6). At the end of the experiment, the highest survival was observed in animals fed red fish protein diets (95.00±2.88%) and the lowest survival was for animals fed blood meal diets (56.67±14.53%). However, a Kruskal-Wallis analysis resulted in no significant differences among diets ($H = 12.84$; $df = 8$; $p = 0.12$).

Similar goblet cell numbers were produced by animals fed white fish meal, red fish meal, soybean, gluten and *Spirulina* protein diets, and these numbers were significantly higher than those produced by animals fed blood meal, meat and bone meal, casein and maize diets (Fig. 4.7; Table 4.3). Goblet cell numbers ranged from 3.11± 0.35 cells per 50 µm (blood protein)

to 24.44±1.98 cells per 50 µm (*Spirulina* protein). A similar trend was found for epithelial thicknesses, where the gluten diet produced animals with the thickest epithelial layers (5.44±0.87 µm) and a blood diet produced animals with the thinnest layers (2.47±0.40 µm).

4.4.3 Proximal analyses

Diets

The results of the proximate analyses for the formulated diets indicate that all the diets generally contained similar protein levels around the 33% protein target (Table 4.1). Thus, crude protein contents ranged from 31.06±2.58% (*Spirulina* protein) to 36.09±1.65% (gluten protein). Crude lipid contents were below 5% for all diets, except *Spirulina*, which could not be maintained below 10.99±1.17%. Ash contents varied from 24.29± 0.04% (meat and bone protein) to 3.26±0.00% (blood protein), due to a greater amount of bone in the meat and bone meals and too few minerals in the blood meal source. Estimated energy and moisture for the diets ranged from 383.4 Kcal/100 g (*Spirulina* protein) to 321.8 Kcal/100 g (blood protein), and from 8.15± 0.00% (gluten protein) to 11.57±0.00 % (maize protein), respectively.

Animals

The proximate analyses of soft bodies of abalone that were raised with various diets for five months are shown in Figure 4.8 and Table 4.4. These results indicate that animals fed different diets had generally similar proximate analysis values. Crude protein contents were the lowest with blood meal diet (48.11±2.89%), while all other treatments resulted in statistically similar values around 66%. Crude lipid contents were relatively low across treatments, ranging from 1.92±0.05% (maize protein) to 4.03±0.03% (*Spirulina* protein). Significant differences were observed in ash content among animals fed different diets, with meat and bone protein diets yielding animals with the lowest percent ash (3.32±0.11%), and *Spirulina* protein diets the highest percent ash (7.01±0.12%). Plant-origin protein diets (*Spirulina* protein, soybean, gluten, maize protein) all had more than 6% ash content, which were significantly higher than fish meal diets (4.68±0.02 and 4.31±0.09% for white and red fish meal, respectively). Moisture content in the soft body (including gonad) samples resulted

in non-significant differences among animals fed the different diets. The moisture contents ranged from 80.65±0.56% with *Spirulina* to 81.95±0.79% with blood protein.

The mean protein gain (MPG) also followed a similar pattern to the growth data (Fig. 4.9, Table 4.4), indicating that white fish meal, red fish meal, casein, soybean, and *Spirulina* protein diets could produce similar high growth for this abalone species. Conversely, blood meal, meat and bone meal, gluten and maize protein diets produced animals with significantly lower MPG. MPG values ranged from -74.67±17.48 mg/abalone (blood protein) to 641.27±56.88 mg/abalone (red fish protein). Only blood protein produced a negative value, which indicates that those animals had muscle loss after the five-month experimental period.

4.4.4 Amino acid profiles

Diet

The amino acid profiles for the nine different diets indicate that white fish meal, red fish meal, and *Spirulina* diets contained the most balanced amino acid profiles, while the other diets were deficient in some amino acids (Table 4.5). For example, compared to the animal-derived protein diets, plant-derived protein diets had relatively low amounts of methionine (0.17-0.21%), but slightly higher than the commercial diet (0.13%). Conversely, these plant-derived protein diets had the highest serine values (9.29-10.07%), which was similar to the commercial diet (10.27%). The commercial diet had high levels of threonine (7.05%) and valine (11.13%), and a moderate amount of all other amino acids compared to the diets used in the feeding trials. With respect to individual amino acids in these diets, there were no significant differences in amino acid content among diets for aspartic acid + aspargine (Asx), glutamic acid +glutamine (Glx), histidine (His), methionine (Met), isoleucine (Ile), leucine (Leu), ornithine (Orn), lysine (Lys), and proline (Pro). The range for these amino acids were 2.03-10.95% for Asx, 3.20-35.52% for Glx, 1.65-10.48% for His, 0.17-11.33% for Met, 1.49-5.13% for Ile, 9.36-23.66% for Leu, 0.06-3.23% for Orn, 0.35-5.49% for Lys, and 0.09-4.09% for Pro. No statistical difference was found in the other amino acid contents across diets (individual amino acids tested by Kruskal-Wallis test, diet as fixed factor; $p < 0.05$).

Animal

The amino acid profiles of abalone soft bodies varied greatly among the different protein diets (Table 4.6). Animals fed the nine diets produced slightly lower amounts of Glx, Leu, Orn and Pro, and a slightly higher amount of Met compared to the initial animals, which were fed the commercial diet. This variation was especially apparent for animals fed the *Spirulina* diet, where significantly low amounts of Glx, Ser, Ala+Tau and Pro, and significantly high amounts of His, Met, Val, Phe, Ile and Lys were observed (Kruskal-Wallis test; $p < 0.05$). In addition, both white and red fish meal diets resulted in significantly low amounts of Glx in the animals' soft bodies. For Gly and Thr, there was an inverse relationship in amino acid content between blood meal, meat and bone, and casein (relatively low for Gly and high for Thr), and soybean, gluten, maize (relatively low for Thr and high for Gly). Finally, no GABA was detected in any of the abalone soft body (including gonad) samples.

The amino acid profiles of old and new shell material also varied among animals fed different diets (Tables 4.7-8). However, no significant differences were observed between old and new shell material for the different amino acids (Kruskal-Wallis tests; shell age as fixed factors; H values ranged from 1.85 to 0.05, p-values ranged from 0.174 and 0.820). In general, His, Gly, Ala+Tau, GABA, and Phe had the greatest variation among diets in the old shell (Table 4.7), and Gly, Arg, Ala+Tau, and GABA had the greatest variation among diets in the new shell (Table 4.8). In addition, the acid/base amino acid ratio (A/B ratio) resulted in no difference between old and new shell material (Kruskal-Wallis; $H = 0.58$, $df = 1$, $p = 0.446$). However, significant differences were found among diets for old (Kruskal-Wallis; $H = 15.97$, $df = 8$, $p = 0.043$) and new (Kruskal-Wallis; $H = 16.37$, $df = 8$, $p = 0.037$) shell material. In the old shell material, the lowest A/B ratio was found in the white fish meal (0.79) and the pre-experiment animals (fed the commercial diet; 0.85), and the highest ratio was found in the casein diet (5.91). For the new shell material, the lowest A/B ratios also were found within the white fish meal (0.91) and the pre-experiment animals (0.41), while the maize (3.44) and soybean (3.09) diets had the highest ratios. Total amino acid amounts in new and old shell materials ranged from 31.82 (meat and bone diet) to 132.25 mg/g (*Spirulina* diet), and 53.85 (meat and bone) to 137.61 mg/g (*Spirulina* diet), respectively.

4.4.5 Fatty acid profiles

Diets

The fatty acid analysis of the feeds resulted in significant differences among the nine diets representing the protein and oil source in the diet (Table 4.9). Significant differences were observed among diets for all fatty acids (individual Kruskal-Wallis; p-values between 0.001 and 0.006). Generally, fish meal diets contained high quantities of polyunsaturated fatty acids and low amounts of saturated fatty acids. For example, white fish meal had the highest amount of eicosadlenoic acid (C20:3n6, 3.42%) and red fish meal contained the highest amounts of linolenic acid (C18:3n3, 2.97%), eicosatrienoic acid (C20:3n3, 0.23%), and EPA (C20:5n3, 22.50%), for a total n-3 fatty acids of 25.92%. Plant-derived diets also had relatively high amounts of poly-unsaturated fatty acids and low amounts of saturated fatty acids. These results reflect the fact that cod liver oil was added to formulate these diets. Thus, soybean, gluten and maize diets contained 71.07, 69.33, and 71.41% unsaturated fatty acids, respectively. The largest contribution to these values was from linolelaidic acid (C18:2n-6) and eicosapentaenoic acid (EPA, C20:5n-3). Blood and meat and bone diets contained more saturated fatty acids and mono-unsaturated fatty acids than the other diets, with blood diets containing the highest amount of saturated fatty acids (53.09%) and meat and bone containing the highest amount of n-9 fatty acids (34.93%). Finally, *Spirulina* diets contained the highest level of total fatty acids (9659mg/ kg diet), palmitic acid (C16:0, 27.27%) and capric acid (C10:0, 1.80%). However, this diet was composed of a more balanced combination of saturated and unsaturated fatty acids, compared with the other diets.

Animal

Comparisons of individual fatty acids among animals fed the different diets also revealed statistical differences (Table 4.10), and significant changes from the commercial diet fed initially, which contained slightly lower total fatty acids. Individual Kruskal-Wallis tests with diet as a fixed factor, and Dunn's multiple comparisons resulted in significant p-values from 0.001 to 0.014. For all animals across diets, there was a general decrease in the amount of n-6, and n-3 fatty acids from 0 to 22.74% and from 0 to 9.98%, respectively. In addition, there was a general increase in n-9 fatty acids from 7.44 to 23.74%. Blood diets were the only diets

which did not contain any n-3 and n-6 unsaturated fatty acids, and animals fed this diet recorded a dramatic decrease in these fatty acids (i.e., they used up all reserves). At the end of the feeding experiments, the main fatty acids recorded from *H. iris* soft bodies were C6:0 (0.70-14.55%), C8:0 (0-15.69%), C14:0 (3.62-11.85%), C16:0 (11.24-41.29%), C16:1 (0-5.94%), C18:0 (0.96-15.02%), C18:1n-9t (1.51-10.95%), C18:1n-9c (1.42-19.60%), C18: 2n-6t (0-15.75%), C18: 2n-6c (0-6.99%), C20:3n-6 (0-12.57%), C24:0 (0-16.07%), and C20:5n-3 (0-9.61%). Finally, fatty acids C16:0 and C18:0 were extremely high, indicating the lack of synthesis of these compounds within their bodies.

4.5 Discussion

The results from the abalone feeding experiments suggest that diets made up of fish meal, casein, soybean, and *Spirulina* protein improve the overall growth, health, and survival of juvenile abalone compared to diets formulated with blood, meat and bone, gluten, and maize as their protein base. In addition, both white and red fish meal diets resulted in better abalone soft body/shell ratios, and animals fed soybean protein diets contained higher gonad and soft body (excluding gonad) indices. The amino acid profiles indicate differences among the diets, but these differences were not always reflected in the animal profiles, indicating that abalone can convert or synthesize amino acids to balance their profiles regardless of the diet composition. Results from the fatty acid profiles suggest that abalone use n-3 and n-6 poly-unsaturated fatty acids as an energy source; they store n-9 mono-unsaturated fatty acids; and may not be able to synthesize C16:0 and C18:0 into longer-chain fatty acids.

4.5.1 Performance of abalone with different dietary proteins

The generally higher growth, health, and survival parameters for animals fed fish meal diets indicate that this protein source generates more muscle tissue and shells containing more aragonite than calcite (i.e., harder shells). Previous studies on other abalone species confirm that fish meals generally yield good animal weight gains. Ogino and Kato (1964) tested diets containing between 15 and 44% fish meal crude protein, and reported a positive linear relationship between the amount of fish meal and growth. Red fish meal (made from *Engraulis capensis* and *Etrumeusteres whiteheadi*) was tested in *H. midae* and showed better

results than casein, *Spirulina* and soy oil cakes (Britz, 1996b). It has been stated that fish meal is the only protein source that can support good abalone growth performance as the sole protein source in a diet, compared to soybean meal and casein, which need other added protein sources to support good growth (Fleming et al., 1996). Thus, fish meal is regularly used as a base protein to which other protein sources may be added to improve growth beyond the basic state. For example, white fish meal was mixed with shrimp meal and defatted soybean meal to improve growth in *H. asinina* (Bautista-Teruel et al., 2003). In the present study, abalone fed fish protein had the highest mean protein gain, which indicates that this diet is readily digested by this abalone species. The high mean protein gain also resulted in the highest soft body (including gonad)/shell (SB/S) ratio, indicating that animals fed fish meal protein diets produced more muscle (higher SB/S rations) than animals fed the other diets. These results suggest that cultivation of *H. iris* for a canned product (meat without shell) may benefit from feeding the stock with a high amount of fish meal protein in their diets. However, fish meal is an expensive protein source. Thus, attempts to find an inexpensive alternative protein source which can achieve similar results are highly relevant for abalone aquaculture.

One potential alternative protein source is soybean protein, which has provided relatively positive results for growth in other abalone species, although significant variations exist among abalone species. For example, Britz (1996b) showed that soybean oil cakes (with 30% protein content) produced slightly faster-growing juvenile *H. midae*, compared to casein protein. However, Uki et al. (1985) compared a variety of protein sources, including soybean oil cake (also with 30% protein content) for their ability to enhance growth of juvenile *H. discus hannai*. While the researchers also found that soybean meal produced good growth, there was no difference in performance compared with casein protein for *H. discus hannai*. In the present study, soybean concentrated protein resulted in good growth for *H. iris*, although not as good as fish meal and casein protein. Soybean concentrated protein is a purified type of protein, from which fibers, lipids and anti-nutrient factors mostly have been removed. This formulation appears to be particularly good for growth of this abalone species, and it could be used effectively as a substitute for fish meal to lower feeding costs of *H. iris*. In addition, the soybean protein diet produced animals with the heaviest gonads and highest gonad/soft body (excluding gonad) indices (GSI) among animals fed different diets. The reason for the greater gonad development with this protein source may be related to the relatively high amount of poly-unsaturated fatty acids (about 47%) provided by the soybean diet. Poly-unsaturated fatty

acids also were found to improve the GSI and reproductive activity in the freshwater swordtail fish, *Xiphophorus helleri* (Lin and Janz, 2006). These results suggest that cultivation of *H. iris* for broodstock purposes may benefit from feeding the stock with a high amount of soybean protein in their diets.

Spirulina protein also produced similar positive growth results as fish meal, soybean and casein protein, and may be used as an alternative inexpensive protein source for *H. iris*. Previous studies have shown high variability in the effectiveness of this protein source with different abalone species (Britz, 1996b; Bautista-Teruel et al., 2003). For example, Britz, (1996b) tested *Spirulina*, soybean oil cakes, torula yeast and casein as protein-based diets, and found that *Spirulina* had the best growth for *H. midae*. Thongrod et al. (2003) compared *Spirulina* and fish meal proteins for their ability to improve growth of *H. asinina*, and found *Spirulina* to have similar results as fish meal. In addition, *Spirulina* also has been used in a spray gel to inoculate grazing surfaces for *H. discus hannai* larvae (Stott et al., 2004). This application has ultimately produced better abalone larval growth than the traditional diatom biofilm. However, in the present study, the lowest SB/S ratios were found in animals fed *Spirulina* protein. These results indicate that the shells of these animals contained higher amounts of protein compared to the shells of animals grown with different diets. In fact, the shell protein level was nearly twice as much as that found in the shells of animals grown with fish meal. A greater contribution of protein in the shell may reflect a lower SB/S ratio (reduced soft body [including gonad] weight). The implications of these findings are that cultivation of *H. iris* for the pearl and shell industry may be fed a diet containing high amounts of *Spirulina*. For example, the new growth shells of animals fed *Spirulina* had strikingly bright red colors,

Casein is one of the most expensive sources of protein for abalone feeds on the market. However, its stable quality and its relatively good growth effects make it a popular protein source in abalone feeds. Casein was first used in formulated feeds by Ogino and Ohta (1963), who investigated various protein sources for their ability to improve abalone growth. Their results indicated that using casein and soybean meal produced the highest growth rates in *H. discus hannai,* compared to other protein sources. Subsequently, casein was used successfully as a basic protein source for a series of comparative nutrient experiments on *H. tuberculata* and *H. discus hannai* (Mai, 1998; Mai et al., 1995a, b; Tan and Mai, 2001). In the present study, casein produced a slightly lower performance on growth, health, and

survival parameters than fish meal, but these results were not statistically significant. However, this trend is supported by other studies on different abalone species (Britz, 1996a; Lopez et al., 1998), and by the fact that casein has a slightly lower feed conversion ratio than fish meal (Britz, 1996b). Based on the acid/basic amino acid ratios, animals grown with casein protein had shells with the highest amount of calcite *versus* aragonite. This indicates that these shells were weaker than the shells of animals fed other protein sources. As a consequence, casein may produce relatively good growth and may be readily available in the market, but its high cost and tendency to produce weaker shells makes it a poor substitute for other protein sources, such as soybean and *Spirulina*.

The other protein sources used in these experiments (meat and bone, maize, wheat gluten, and blood protein) did not perform well across growth and health parameters for *H. iris*. Meat and bone meal has not been tested as a protein source for formulated abalone feeds until now, but is commonly used in fish aquaculture as a good substitute for fish meal (Allan, 1979; Lovell, 1989; Shimeno et al., 1993a, b; Tacon, 1994). This protein source was tested in this experiment because it has a relatively low cost. However, its relatively poor performance and high amount of dietary ash (24.3% mainly from bone) make it an undesirable protein source for abalone feeds. The high amount of ash not only reduced the physical stability of the pellets, but ash is indigestible and tends to dilute the nutrients in the diet (Robina et al., 1997). Blood protein also has not been tried as the main protein source in abalone feeds, although it often is used as an additional protein source. For example, Flemming et al. (1996) reported a small proportion (4.5%) of blood meal used in commercial diets for *H. rubra* in Australia. In the present study, blood protein produced the lowest *H. iris* growth, resulting in less than half the growth of abalone fed fish meal. In addition, a negative mean protein gain for animals fed this diet indicates that *H. iris* may not be able to digest blood protein. Similar results were found for the shrimp, *Penaeus californiensis*, when fed a 5-10% blood protein replacement of a fish meal diet (Brand and Colvin, 1977). Marichal et al. (2000) suggested that different manufacturing drying temperatures (oven-dried at 100-160 ºC or vacuum-dried at 70 ºC) may result in different availability values through physicochemical modifications (denaturation) and linkages with other substances (i.e., Maillard reaction). In this study, the blood protein used was oven-dried at high temperature (100-160 ºC). However, further studies will need to be conducted to clearly elucidate the effect of manufacturing methodology on the digestibility of this kind of protein. Regardless, blood protein appears to be unsuitable for feeding *H. iris* as a sole protein source.

Overall, maize and wheat gluten proteins also resulted in poor growth and health aspects of juvenile *H. iris* cultivation. While maize protein resulted in slightly better performance than gluten protein, neither protein source appeared to be appropriate for *H. iris* as a sole protein source. Similar results were found for maize protein in the diet of *H. discus hannai* juveniles (Uki et al., 1985), and the use of gluten as a binding agent has been successfully tested for *H. fulgens* feeds (Guzmán and Viana, 1998). However, further research will need to be conducted to examine the use of these types of protein as binding agents in *H. iris* diets.

4.5.2 Amino acid profiles

The amino acid profiles in the soft bodies of juveniles fed different diets are variable among diets. However, the general profiles are consistent with other abalone species (Mai et al., 1994), although argentine is higher than reported for *H. tuberculata* (8.01%) and *H. discus hannai* (9.39%). Regardless of these differences, the nine diets used in these experiments did not appear to cause dramatic deficiencies in any one amino acid. Furthermore, low methionine levels in the plant protein diets (soybean, gluten, maize, and *Spirulina*) were expected to result in amino acid deficiencies in soft body (including gonad), but these deficiencies were not observed. In fact, soybean diets provided animals with the lowest methionine concentrations of any diet. For aquatic animals, such as fish, methionine is an essential amino acid which takes part in protein synthesis and other important physiological functions, as well as an indispensable amino acid for normal growth (Lovell, 1989). Thus, low amounts of methionine in the diet would be expected to result in poor growth (Walton et al., 1982; Rumsey et al., 1983; Cowey, 1992). For example, a methionine deficiency in soft body tissues was found in the abalone, *H. asinina*, and decreased growth after the fish meal diet was replaced with 35% defatted soybean and 20% *Spirulina* protein (Bautista-Teruel et al., 2003). The reasons for this discrepancy may be due to a lower methionine requirement of *H. iris*, which results in a much wider range of food sources that can sustain good growth in this species. Similar observations were made with tilapia (*Sarotherodon mossambicus*), which were fed a diet of a variety of plant material, and the fish still maintained their high growth rates (Jackson and Capper, 1982).

Another interesting result was that GABA was not detected in either diet or animal soft bodies, but appeared in both new and old shells of all animals. This result suggests that GABA can be synthesized in *H. iris*, and that it plays a role in shell formation. Studies with mammal species have shown that GABA is directly biosynthesized from L-glutamate by the work of glutamic acid decarboxylase (Bene et al., 2007). In gastropods, GABA is only synthesized within neuron bundles (Cooke and Gelperin, 1988; Hernadi, 1994), and the epithelial cells of the cephalic and epipodial tentacles (Wanichanon et al., 2004). GABA has been found in the mucus of *H. rubra*, *H. asinina*, and *H. diversicolor* (Laimek et al., 2008). However, the 1 to 5% GABA found in the shells of *H. iris* represents the first report of this amino acid in this species and within gastropod shell material, although its function is still unclear.

4.5.3 Fatty acid profiles

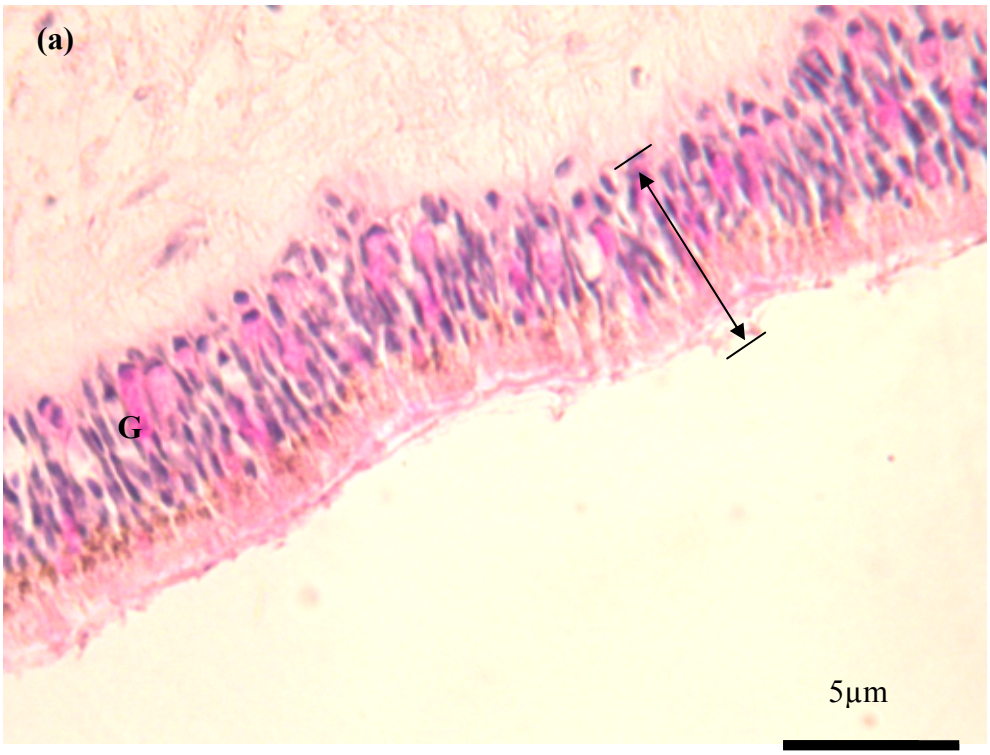
In this study, the amount of lipids within the different diets was controlled at about 5%, except for the *Spirulina* diet, which could not be formulated at less than 10% (this microalga itself contains a high amount of oil). In addition to *Spirulina*, meat and bone was the only other diet to contain its own lipid source (all other diets contained some amount of fish oil to achieve a standard 5% lipid content). However, lipids were found to not significantly affect the growth of *H. iris*. In fact, none of the treatments resulted in animals with more than 4% crude lipid content after the feeding trials, even though they had been fed over 5% in every case. Furthermore, the nearly double amount of oil in the *Spirulina* diet, compared to the other diets, also resulted in only 4% crude lipid in the animals fed that diet. It is well known that some fatty acids stimulate growth, while others are readily burned or stored (Mai et al., 1996). However, no particular fatty acid was found to dramatically enhance growth of this abalone species. Conversely, poly-unsaturated fatty acids have been found to stimulate growth of *H. discus hannai* (Uki et al., 1986a, b; Mai et al., 1996). The reason for this discrepancy is likely to be that previous researchers have used higher amounts of PUFA in the diet, while in this study the aim was to maintain a generally low and similar amount of lipids across diets.

While no significant growth differences were found due to variations in fatty acid profiles, some distinctive differences were observed in the fatty acid profiles after the feeding

experiments. High levels of palmitic acid (C16:0) and stearic acid (C18:0) were found in animals across diets, indicating an accumulation of this fatty acid. This trend was particularly obvious in animals fed blood protein (about 41 and 15% for C16:0 and C18:0, respectively), which appeared to be starving at the end of the experiment. Previous studies have suggested that *H. discus hannai* have the ability to synthesize 20:5n-3, 20:4n-6 and 22:6n-3 (PUFA) from C16 and C18 PUFA (Xu et al., 2004). However, the results from this study suggest that if this synthesis takes place in *H. iris*, it is not very active under the present feeding conditions (i.e., high levels of C16:0, and low levels of 20:5n-3, 20:4n-6 and 22:6n-3 after feeding trials). Instead, animals across all diets appeared to have used most of the PUFA in the diets for growth, and in the case of animals fed blood protein diets, they began to starve. PUFA, such as EPA (20:5n-3), 20:4n-6 and 22:6n-3, and other n-3 and n-6 amino acids have been found to play a prominent role in the growth of *H. discus hannai* (Uki et al., 1986a; Mai et al., 1996).

In conclusion, fish meal diets performed better than other diets for the growth, health and survival of *H. iris* juveniles in the present study. Soybean meal and *Spirulina* appear to be good alternative protein sources, which may be able to be used to completely replace the use of fish meal in *H. iris* diets. Deficiencies of methionine in soybean diets did not affect the growth and survival of *H. iris*. Different protein sources in the diets affected the amino acid profiles in shell material, which may result different biomineralization processes, such as calcite and aragonite incorporation in the shell matrix.

Figure 4. 1. Histological section of an abalone juvenile fed with (a) *Spirulina* protein and (b) blood meal protein diets for a five-month period. Periodic Acid-Schiff stain and Mayer's hematoxylin were applied to sections for counter-staining the tissues. Goblet cells are denoted by the letter "G", and the arrows indicate epithelial thickness measurements.



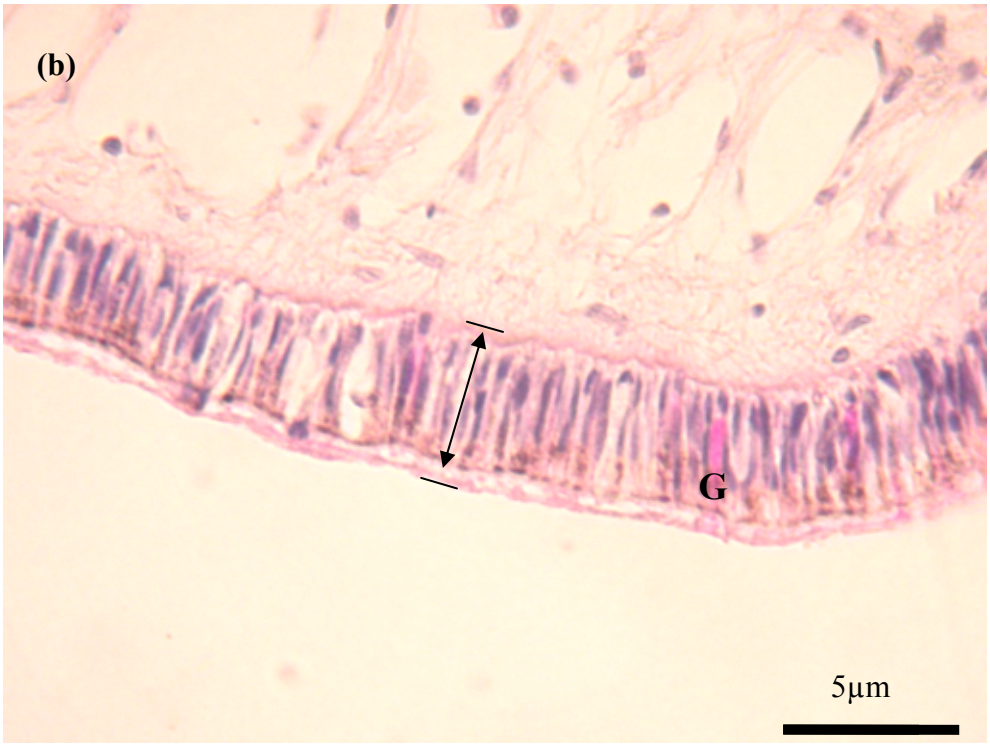


Figure 4. 2. (a) Maximum shell length growth, (b) wet shell weight and (c) dry shell weight after the five-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

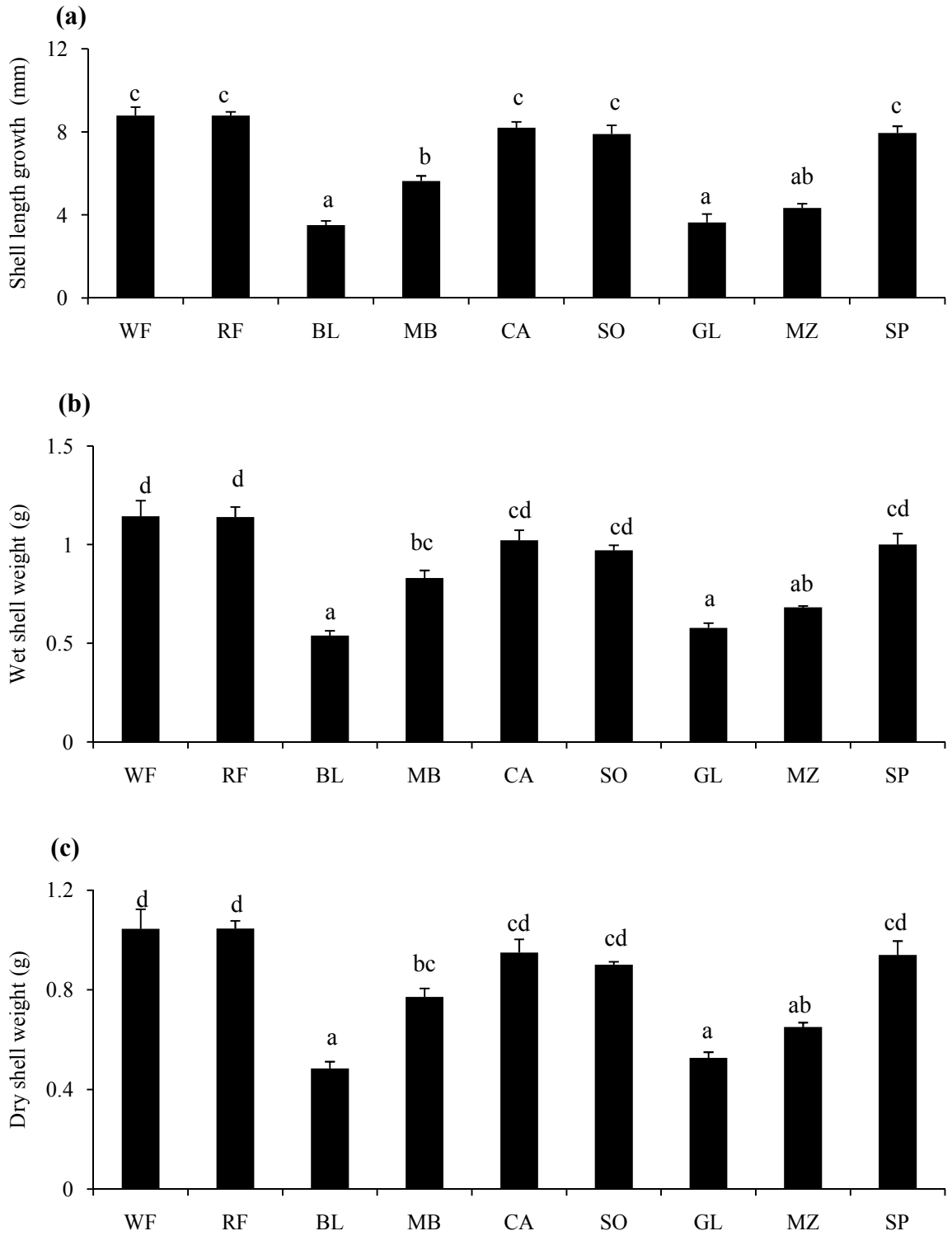


Figure 4. 3. **(**a) Total wet weight gain, (b) wet weight of soft bodies and (c) dry weight of soft bodies after the five-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

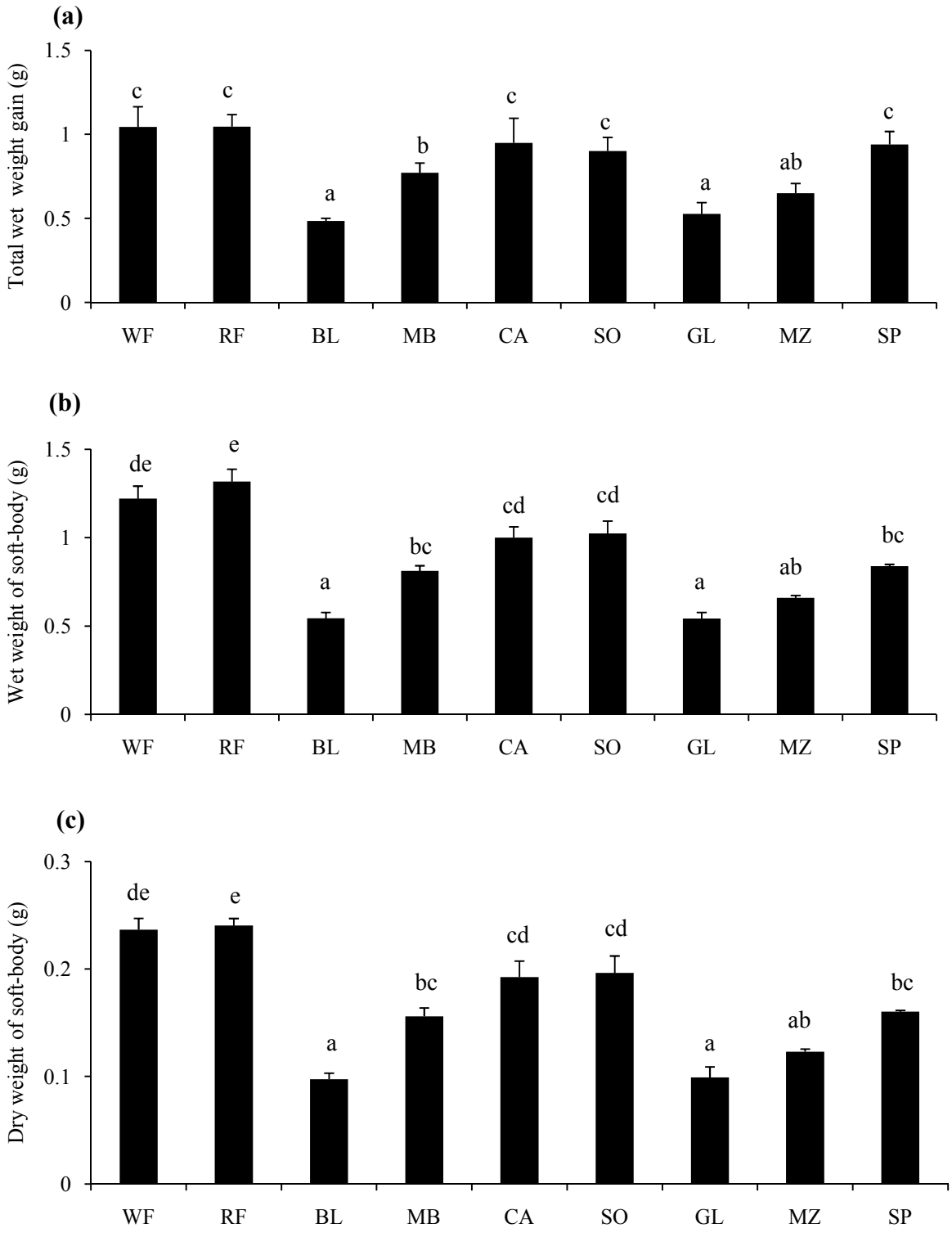


Figure 4. 4. (a) Wet and (b) dry soft body(including gonad / shell ratios after the five-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

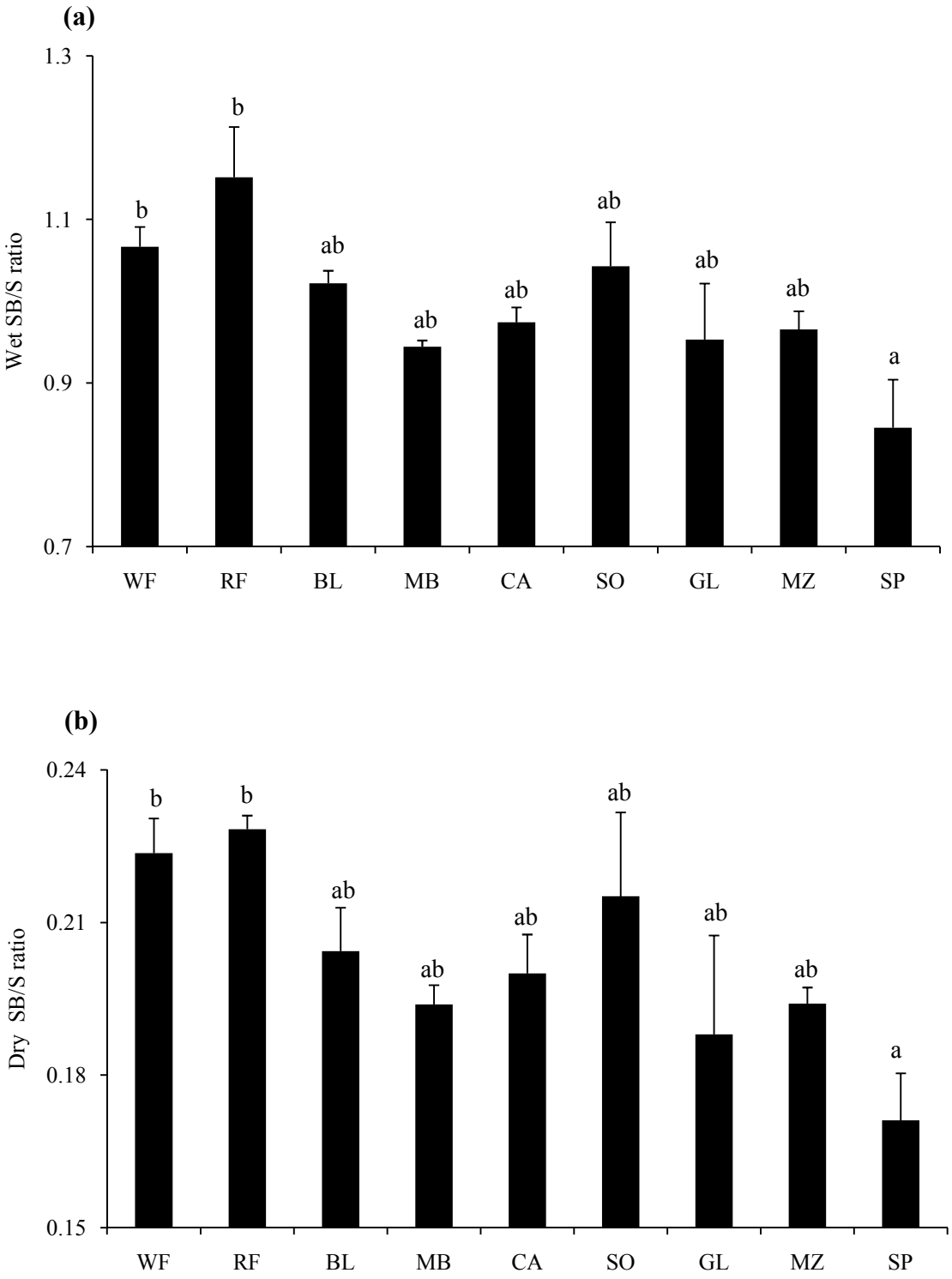


Figure 4. 5. (a) Gonad wet weights and (b) gonad soft body (excluding gonad) indices (GSI) of soft bodies after the five-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

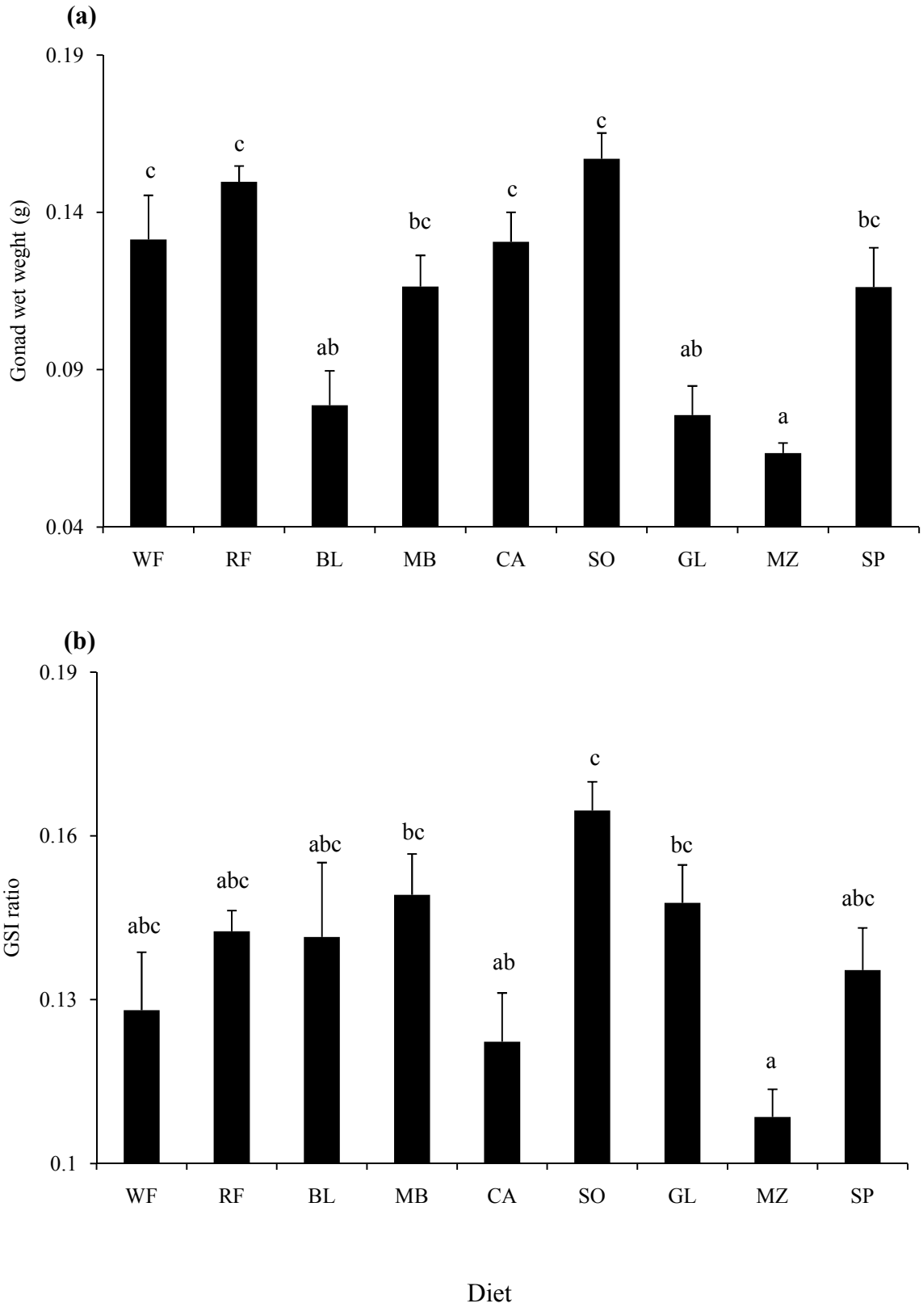


Figure 4. 6. Percent survival of abalone juvenile on different diets over the five-month experimental period. Data are presented as means of three replicate experimental tanks.

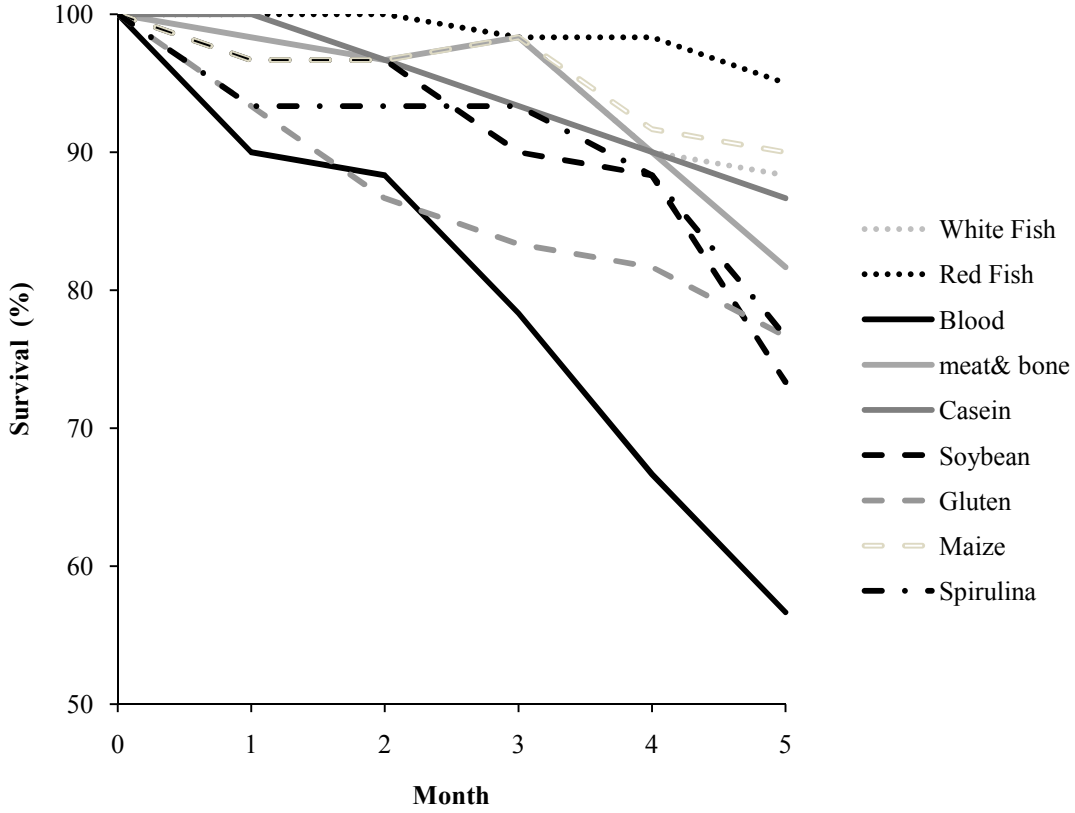


Figure 4. 7. (a) Goblet cell number over a 5µm epithelial layer section, and (b) epithelial thickness of animals after the five-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

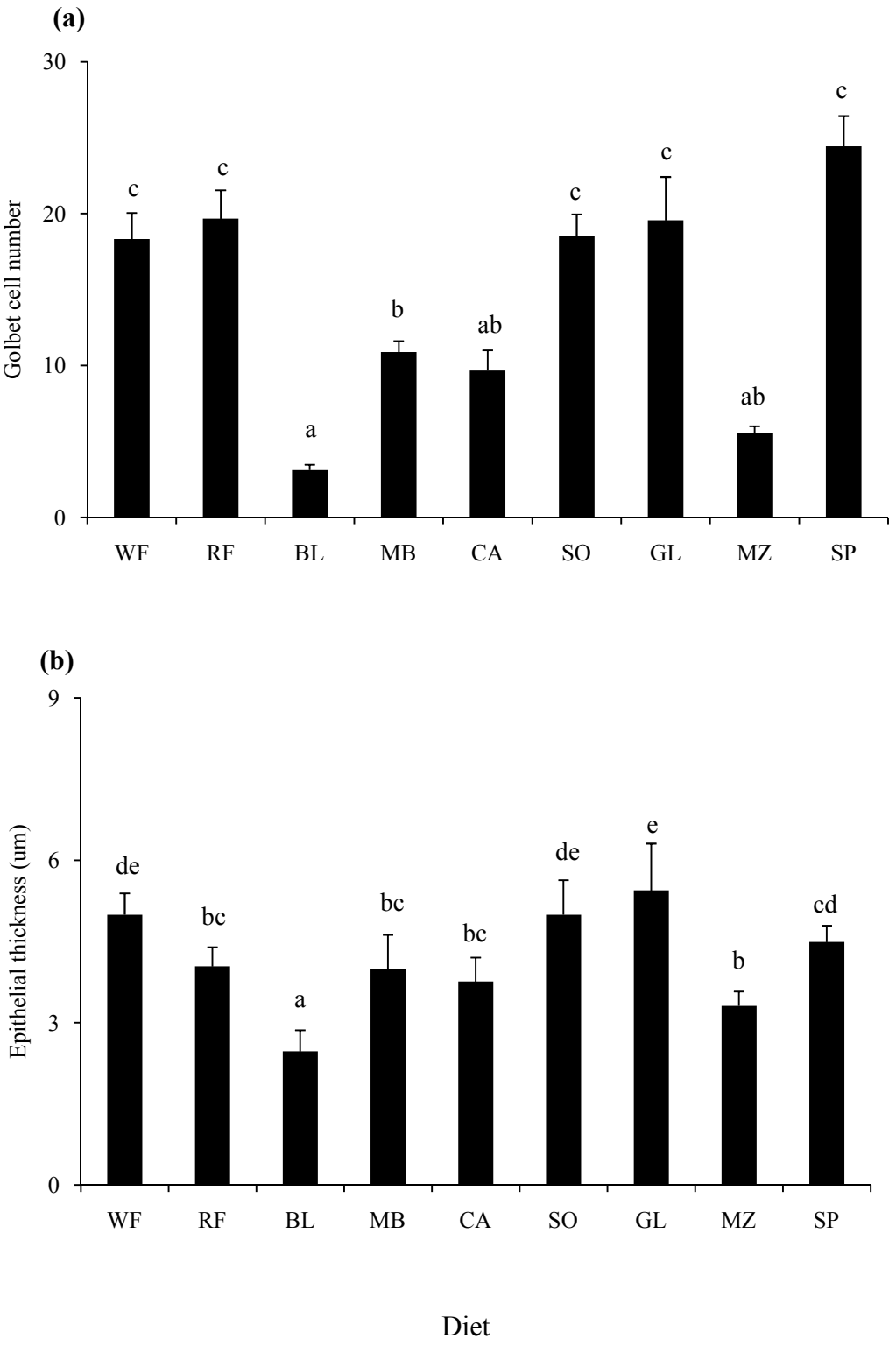


Figure 4. 8. Proximate analyses of diets. Percent (a) crude protein, (b) crude lipid (c) ash, and (d) moisture of soft bodies after the five-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

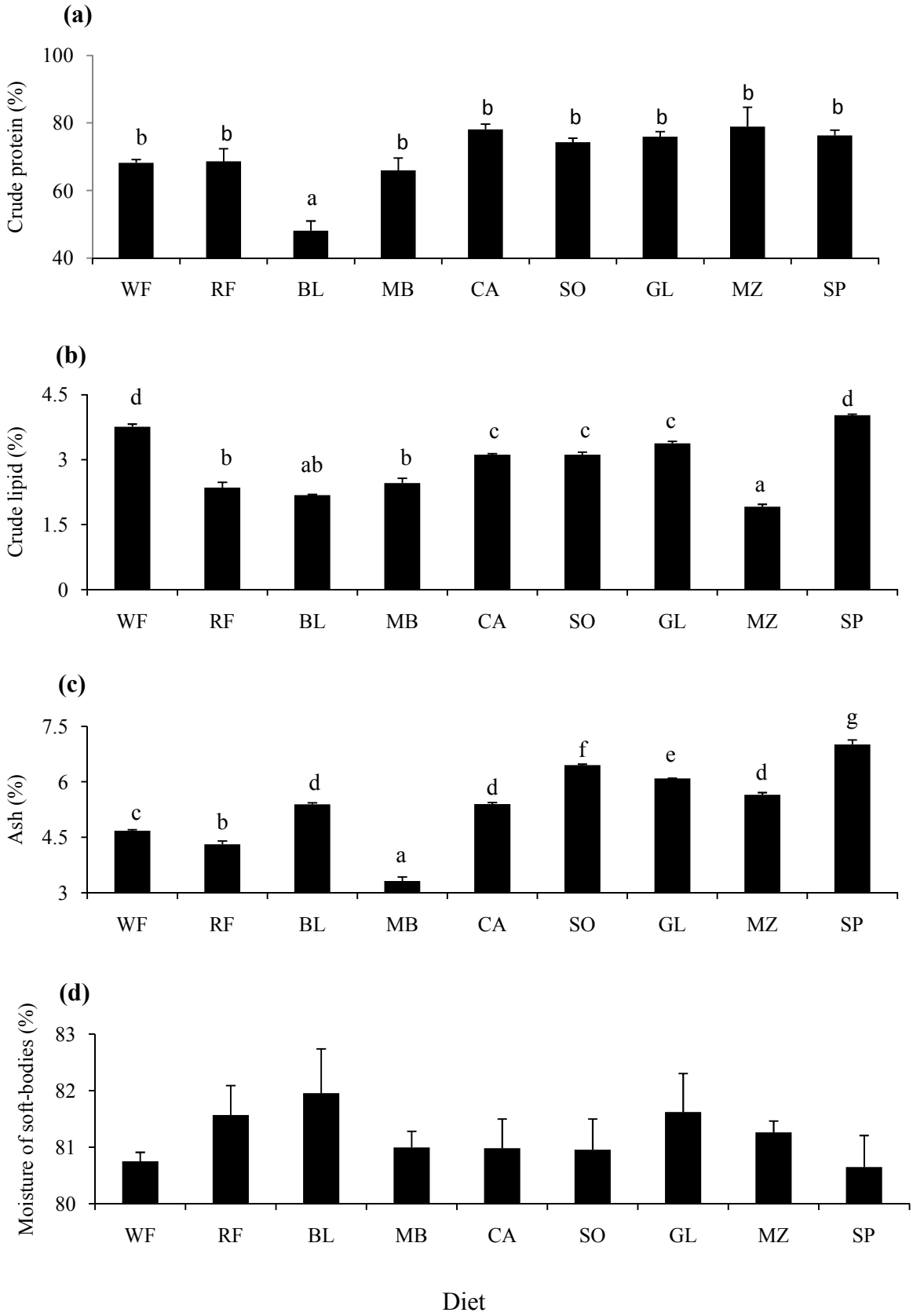


Figure 4. 9. Mean protein gain of abalone after the five-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

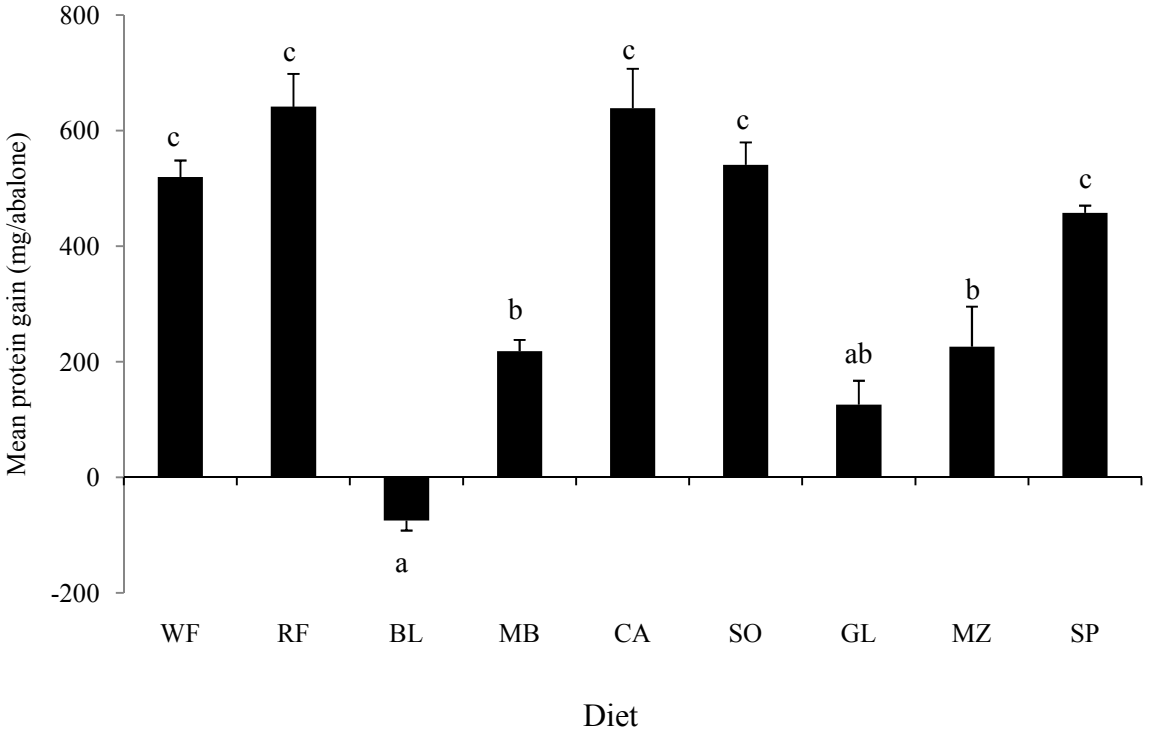


Table 4. 1. Recipes for nine experimental diets with different protein sources, and proximate analyses for each diet. Data are presented as means ± SD of three replicate experimental samples. The crude energy for 100 g of diet was calculated from the sum of mean gross energy per gram of carbohydrate (maintained constant at 40% in the diet with 4 kcal energy per gram), lipid (9 kcal) and protein (4 kcal) in each diet (Brett and Grove, 1979).

Diet	White fish meal[a] (WF)	Red fish meal[a] (RF)	Blood meal[a] (BL)	Meat and bone meal[a] (MB)	Casein[a] (CA)	Soybean concentrate[a] (SO)	Wheat gluten[a] (GL)	Maize gluten[a] (MZ)	*Spirulina*[a] (SP)
Protein (g/100g diets)	42.40	40.50	29.70	52.00	31.90	54.90	35.80	42.50	49.60
Cellose[b] (g/100g diets)	14.10	16.50	22.40	-	20.2	-	16.7	11.9	7.40
Starch[c] (g/100g diets)	40.0	40.00	40.00	40.00	40.0	40.0	40.0	40.0	40.00
Lipid[d] (g/100g diets)	0.50	-	4.90	-	4.90	4.10	4.50	2.60	-
Vitamine[e] (g/100g diets)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Minerial Mix[f] (g/100g diets)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Proximate analysis									
Crude protein (%)	35.98±2.02	34.68±2.74	31.25±1.72	32.40±1.83	31.76±1.25	34.74±2.40	36.09±1.65	36.00±4.5	31.06±2.58
Crude lipid (%)	4.82±0.02	4.41±0.41	4.09±0.09	4.04±0.63	4.58±0.09	4.43±0.21	4.17±0.03	3.88±0.08	10.99±1.17
Ash content (%)	11.61±0.01	10.29±0.00	3.26±0.00	24.29±0.04	4.04±0.01	6.69±0.00	5.67±0.04	5.61±0.04	8.52±0.00
Moisture (%)	8.31±0.00	9.02±0.00	10.17±0.00	11.19±0.00	7.79±0.00	7.95±0.00	8.15±0.00	11.57±0.00	10.29±0.00
Crude energy (Kcal/100g)	347.28	338.39	321.82	343.96	328.27	338.83	341.51	338.92	383.14
Average pellet weight (g)	0.18	0.25	0.23	0.34	0.19	0.25	0.39	0.26	0.26

a. white and red fish meal supplied by Sealord, Nelson (NZ) LTD. Casein supplied by Anchor; the meat and bone meal, and the blood meal supplied by Affco (NZ) Limited, wheat gluten, maize gluten and soybean concentrates from J. C. Sherratt & Co. Ltd.; Spirulina from Superfood Spirulina Ltd.

b. from commercial product "Just-Fiber ™".

c. modified starch (Firm-MIX ™) used for binding pellets from New Zealand Starch LTD.

d. cold-filter cod liver oil (Healther™, NZ).

e. Vitamin mix (Shin- Chan™, Taiwan) per g content: Riboflavin 10 mg, PABA 40 mg, Pyridoxine HCl 4 mg, Niacin 80 mg, Ca pantothenate 20 mg, Inositol 400 mg, Ascorbic acid 400 mg, Biotin 1.2 mg, Vitamin E 45 mg, Menadione 8 mg, Vitamin B_{12} 18 mg, Vitamin A 10000 I.U., Vitamin D 200 I.U., Ethoxyquin 40 mg, Folic acid 3 mg, Thiamin HCl 12 mg.

f. Mineral mixture g/kg mixture: NaCl (BDH) 10 g, $MgSO_4 \cdot 7H_2O$ (BDH) 10 g, $NaH_2PO_4 \cdot 2H_2O$ (BDH) 150 g, KH_2PO_4 (BDH) 25 0g, K_2HPO_4 (BDH) 320 g, $Ca(H_2P0_4)_2 \cdot H_2O$ (Merck) 200 g, Fe-lactate (SIGMA) 25 g, Ca-lactate (SIGMA) 35 g, $ZnS04 \cdot 7H_2O$ (BDH) 3.53 g, $MnS0_4 \cdot 4\ H_2O$ (BDH) 1.62 g, $CuSO_4 \cdot 5H_2O$(BDH) 310 mg, $CoC1_2 \cdot 6H_2O$ (Merck) 10 mg, KIO_3 (Merck) 30 mg.

Table 4. 2. One-way ANOVA results of growth parameters (maximum shell length, total weight, wet and dry soft body (including gonad) weights, wet and dry shell weights, wet and dry SB / S ratios, gonad wet weights and gonad and soft body (excluding gonad) indices for abalone juveniles after the five-month experimental period.

	Maximum shell length				*Total weight*		
Source	df	MS	F	p	df	MS	F	p
Diet	8	14.98	51.03	0.001	8	1.00	46.02	0.001
Error	18	0.29			18	0.02		
Total	26				26			
	Wet soft body weight				*Dry soft body weight*		
Source	df	MS	F	p	df	MS	F	p
Diet	8	0.23	31.25	0.001	8	0.01	31.52	0.001
Error	18	0.01			18	0.00		
Total	26				26			
	Wet shell weight				*Dry shell weight*		
Source	df	MS	F	p	df	MS	F	p
Diet	8	0.16	25.93	0.001	8	0.14	25.72	0.001
Error	18	0.01			18	0.01		
Total	26				26			
	Wet SB / S				*Dry SB / S*		
Source	df	MS	F	P	df	MS	F	p
Diet	8	0.02	4.12	0.010	8	0.00	3.13	0.020
Error	18	0.01			18	0.00		
Total	26				26			
	Gonad wet weight				*Gonad soft body index (GSI)*		
Source	df	MS	F	p	df	MS	F	p
Diet	8	0.01	11.33	0.001	8	0.00	4.03	0.001
Error	18	0.00			18	0.00		
Total	26				26			

Table 4. 3. One-way ANOVA results of goblet cell number and epithelial thickness after the five-month experimental period.

	Goblet cell number				*Epithelial thickness*			
Source	df	MS	F	p	df	MS	F	p
Diet	8	481.9	20.82	0.001	8	1.95	29.57	0.001
Error	72	23.1			72	0.07		
Total	80				80			

Table 4. 4. One-way ANOVA results of proximate analysis and mean protein gains after the five-month experimental period.

		Crude protein content				*Crude lipid content*		
Source	df	MS	F	p	df	MS	F	p
Diet	8	276.7	10.58	0.001	8	1.61	115.41	0.001
Error	18	26.1			18	0.01		
Total	26				26			
		Ash content				*Moisture content*		
Source	df	MS	F	p	df	MS	F	p
Diet	8	3.85	273.42	0.001	8	0.59	0.73	0.670
Error	18	0.01			18	0.79		
Total	26				26			

		Mean protein gain		
Source	df	MS	F	p
Diet	8	189725	32.29	0.001
Error	18	5875		
Total	26			

Table 4. 5. Amino acid profiles of nine diets (mg /100 mg dietary protein). Data are presented as means of three replicate experimental samples. The nine protein sources are WF = White fish meal, RF = Red fish meal, BL = Blood meal, MB = Meat and bone meal, CA = Casein, SO = Soybean concentrate, GL = Gluten, MZ = Maize gluten and SP =*Spirulina*. The commercial diet was used prior to the start of the experiment. Superscripts represent significant differences (Dunn's tests; $p < 0.05$).

Diet	WF	RF	BL	MB	CA	SO	GL	MZ	SP	Commercial
Asx	7.19^{ab}	10.95^{b}	2.03^{a}	9.02^{ab}	5.82^{ab}	9.25^{ab}	4.14^{ab}	6.46^{ab}	8.89^{ab}	6.77
Glx	13.75^{ab}	15.16^{ab}	3.20^{a}	15.69^{ab}	17.16^{ab}	12.84^{ab}	35.52^{b}	13.71^{ab}	14.84^{ab}	10.35
Ser	8.67	7.70	5.39	7.58	6.38	9.52	10.07	9.29	7.46	10.27
His	2.27^{ab}	3.44^{ab}	10.48^{b}	1.93^{ab}	1.65^{a}	5.60^{ab}	3.52^{ab}	3.98^{ab}	3.09^{ab}	2.53
Gly	8.21	0.45	6.26	0.43	2.55	4.01	2.54	0.96	3.70	4.14
Thr	7.97	6.45	7.93	5.81	4.26	7.58	4.65	4.18	7.12	6.29
Arg	8.21	7.22	8.61	9.40	2.23	4.57	3.37	9.70	7.53	3.66
Ala+ Tau	10.54	8.32	10.58	12.17	7.41	13.41	1.87	4.51	11.96	6.28
GABA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tyr	3.51	4.32	4.36	3.90	5.58	6.00	3.61	5.52	3.81	7.05
Met	2.82^{ab}	2.69^{ab}	1.73^{ab}	0.30^{ab}	11.33^{b}	0.17^{a}	0.23^{ab}	0.21^{ab}	0.58^{ab}	0.13
Val	7.22	8.09	11.09	10.05	7.56	5.92	7.32	6.51	8.72	11.13
Phe	4.90	7.06	9.26	6.18	6.14	6.44	6.88	7.57	5.04	8.67
Ile	4.32^{ab}	4.78^{ab}	1.49^{a}	4.54^{ab}	4.70^{ab}	3.67^{ab}	4.16^{ab}	3.31^{ab}	5.13^{b}	6.66
Leu	9.36^{a}	12.00^{ab}	11.98^{ab}	10.66^{ab}	11.10^{ab}	10.14^{ab}	10.29^{ab}	23.66^{b}	10.74^{ab}	15.55
Orn	0.10^{ab}	0.38^{ab}	0.12^{ab}	0.67^{ab}	3.23^{b}	0.26^{ab}	0.72^{ab}	0.06^{a}	0.35^{ab}	0.13
Lys	0.96^{ab}	1.00^{ab}	5.49^{b}	1.66^{ab}	2.91^{ab}	0.63^{ab}	1.14^{ab}	0.35^{a}	1.03^{ab}	0.41
Pro	0.09^{a}	1.16^{ab}	0.71^{ab}	1.51^{ab}	4.09^{b}	3.30^{ab}	1.68^{ab}	2.86^{ab}	0.71^{ab}	2.59

Table 4. 6. Amino acid profiles of soft body (including gonad) tissues (mg /100 mg protein). Data are presented as means of three replicate experimental samples. The nine protein sources are WF = White fish meal, RF = Red fish meal, BL = Blood meal, MB = Meat and bone meal, CA = Casein, SO = Soybean concentrate, GL = Gluten, MZ = Maize gluten and SP =*Spirulina*. The commercial diet was used prior to the start of the experiment. Superscripts represent significant differences (Dunn's tests; $p < 0.05$).

Diet	WF	RF	BL	MB	CA	SO	GL	MZ	SP	Commercial
Asx	5.36	5.93	5.76	6.80	6.09	6.47	7.69	5.74	4.01	7.15
Glx	5.77^{ab}	5.61^{ab}	7.72^{b}	7.45^{b}	7.66^{b}	7.51^{b}	7.73^{b}	6.96^{ab}	3.89^{a}	9.39
Ser	15.69^{bc}	16.10^{bc}	14.23^{ab}	13.69^{ab}	16.03^{bc}	17.29^{c}	17.22^{c}	16.08^{bc}	12.83^{a}	15.40
His	1.88^{abc}	1.97^{abc}	1.53^{ab}	1.48^{a}	1.65^{ab}	1.75^{ab}	1.63^{ab}	2.11^{bc}	2.45^{c}	1.63
Gly	6.25^{b}	7.63^{b}	0.74^{a}	0.57^{a}	0.90^{a}	6.96^{b}	7.22^{b}	7.34^{b}	3.14^{a}	6.66
Thr	9.93^{a}	9.48^{a}	24.49^{c}	21.25^{bc}	21.26^{bc}	9.83^{a}	9.64^{a}	10.74^{a}	15.73^{ab}	8.39
Arg	16.21	15.63	11.48	13.40	13.94	14.63	13.54	16.65	15.99	12.40
Ala+ Tau	5.54^{ab}	5.93^{b}	4.65^{ab}	5.14^{ab}	5.39^{ab}	5.62^{ab}	4.59^{ab}	5.58^{ab}	4.22^{a}	5.70
GABA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tyr	2.23	2.05	1.54	1.36	1.87	1.98	1.91	2.42	1.58	1.85
Met	2.26^{ab}	2.10^{ab}	1.69^{a}	1.83^{ab}	1.76^{a}	1.92^{ab}	1.94^{ab}	2.12^{ab}	3.19^{b}	1.56
Val	4.17^{ab}	3.61^{ab}	3.18^{a}	3.22^{a}	3.64^{ab}	4.00^{ab}	4.14^{ab}	4.46^{b}	5.75^{c}	3.32
Phe	5.85^{a}	5.57^{a}	4.78^{a}	5.29^{a}	4.89^{a}	5.47^{a}	5.72^{a}	6.12^{a}	8.11^{b}	5.58
Ile	3.38^{c}	2.74^{abc}	2.68^{ab}	2.59^{a}	2.67^{ab}	2.99^{abc}	3.28^{bc}	3.21^{abc}	4.40^{d}	2.64
Leu	10.95^{b}	10.00^{ab}	10.36^{ab}	10.20^{ab}	9.20^{a}	9.97^{ab}	10.29^{ab}	10.50^{ab}	9.20^{ab}	11.65
Orn	1.01^{ab}	1.13^{ab}	2.20^{b}	2.20^{b}	0.59^{a}	0.99^{ab}	0.91^{ab}	1.24^{ab}	0.60^{a}	2.70
Lys	3.27^{ab}	4.26^{ab}	2.53^{ab}	3.16^{ab}	2.12^{a}	2.24^{a}	2.15^{a}	3.25^{ab}	4.76^{b}	3.47
Pro	0.27^{ab}	0.26^{ab}	0.46^{b}	0.37^{b}	0.36^{b}	0.39^{b}	0.40^{b}	0.37^{b}	0.13^{a}	0.51

Table 4. 7. Amino acid profiles of new shell material (mg/100mg shell protein). Data are presented as means of three replicate experimental samples. The nine protein sources are WF = White fish meal, RF = Red fish meal, BL = Blood meal, MB = Meat and bone meal, CA = Casein, SO = Soybean concentrate, GL = Gluten, MZ = Maize gluten and SP =*Spirulina*. The commercial diet was used prior to the start of the experiment. Superscripts represent significant differences (Dunn's tests; $p < 0.05$).

Diet	WF	RF	BL	MB	CA	SO	GL	MZ	SP	Commercial
Asx	17.39	18.18	20.73	24.84	14.85	16.95	30.24	20.78	19.94	11.95
Glx	8.37	7.27	11.24	8.54	12.25	14.16	7.62	14.15	8.99	5.87
Ser	14.58	18.12	17.31	20.71	20.80	22.46	17.04	19.87	19.22	14.32
His	12.00	15.01	8.50	2.96	8.06	4.27	4.75	1.80	8.83	15.92
Gly	4.46^{c}	4.28^{bc}	2.53^{abc}	2.02^{a}	2.31^{abc}	2.14^{ab}	2.69^{abc}	1.94^{a}	2.09^{ab}	2.57
Thr	5.74	6.09	3.72	3.10	7.61	8.32	4.25	8.30	7.55	4.89
Arg	16.77^{b}	13.21^{ab}	12.84^{ab}	14.63^{ab}	10.17^{ab}	8.21^{a}	11.47^{ab}	8.54^{a}	12.86^{ab}	26.08
Ala+ Tau	2.40^{ab}	2.30^{a}	3.00^{abc}	3.57^{a}	3.39^{bc}	3.62^{abc}	2.78^{ab}	3.11^{abc}	3.28^{c}	2.44
GABA	3.34	2.79	2.42	1.50	2.98	2.90	1.63	2.39	3.23	4.56
Tyr	1.41	1.01	1.77	1.90	1.94	2.11	1.64	2.10	1.50	1.14
Met	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Val	2.75	2.20	3.71	3.79	3.83	4.07	3.95	4.73	2.61	1.92
Phe	5.01	4.00	5.18	5.61	5.72	5.35	5.00	5.80	4.68	3.11
Ile	1.33	1.00	1.58	1.47	1.42	1.38	1.49	1.72	1.96	0.70
Leu	3.72	3.06	4.22	4.75	3.97	3.78	4.54	4.26	2.40	2.68
Orn	0.00	0.00	0.49	0.00	0.00	0.00	0.00	0.00	0.21	0.00
Lys	0.57	0.97	0.55	0.27	0.54	0.18	0.71	0.41	0.47	1.48
Pro	0.15	0.50	0.22	0.33	0.17	0.12	0.20	0.10	0.18	0.37
A/B ratio	0.91^{ab}	0.92^{ab}	1.85^{ab}	1.95^{ab}	2.01^{ab}	3.09^{b}	2.44^{ab}	3.44^{b}	1.32^{ab}	0.41^{a}
TAA (mg /g shell)	48.01	34.32	87.33	31.81	51.64	47.82	40.15	36.42	132.23	35.01

Table 4. 8. Amino acid profiles of old shell material (mg/100mg shell protein). Data are presented as means of three replicate experimental samples. The nine protein sources are WF = White fish meal, RF = Red fish meal, BL = Blood meal, MB = Meat and bone meal, CA = Casein, SO = Soybean concentrate, GL = Gluten, MZ = Maize gluten and SP =*Spirulina*. The commercial diet was used prior to the start of the experiment. Superscripts represent significant differences (Dunn's tests; $p < 0.05$).

Diet	WF	RF	BL	MB	CA	SO	GL	MZ	SP	Commercial
Asx	12.23	15.99	19.95	23.38	19.84	21.50	18.84	16.60	23.32	13.42
Glx	9.59	7.61	14.39	8.61	15.65	10.74	6.82	14.07	11.91	10.36
Ser	23.59	19.05	21.33	20.67	23.24	22.19	17.85	19.54	17.71	17.70
His	12.60^{c}	5.20^{abc}	4.98^{abc}	1.45^{ab}	1.87^{ab}	0.54^{a}	10.70^{bc}	0.50^{a}	6.58^{abc}	13.24
Gly	3.56^{ab}	5.64^{b}	2.28^{ab}	0.75^{a}	1.48^{a}	1.67^{a}	4.26^{ab}	1.40^{a}	2.13^{ab}	2.60
Thr	4.16	10.80	4.41	5.38	6.99	8.04	5.61	5.50	3.65	5.82
Arg	15.69	14.13	7.46	16.07	6.24	8.84	14.32	20.02	13.01	13.69
Ala+ Tau	2.38^{a}	2.15^{a}	2.56^{ab}	3.69^{b}	3.19^{ab}	3.38^{ab}	3.19^{ab}	3.15^{ab}	2.74^{ab}	3.03
GABA	2.92^{ab}	4.84^{b}	2.88^{ab}	1.42^{a}	2.49^{ab}	2.38^{ab}	1.62^{a}	1.93^{a}	1.86^{a}	2.95
Tyr	1.83	2.39	2.97	2.31	3.22	3.41	2.01	2.73	2.74	2.12
Met	0.08	0.25	0.11	0.20	0.31	0.28	0.42	0.24	0.22	0.25
Val	2.60	2.74	4.04	3.82	4.00	4.30	3.35	3.92	3.61	3.37
Phe	3.99^{a}	3.91^{a}	5.32^{ab}	5.37^{ab}	5.59^{ab}	6.10^{b}	4.65^{ab}	4.69^{ab}	4.42^{ab}	4.60
Ile	0.96	1.08	1.53	1.41	1.42	1.66	1.94	1.49	1.21	1.33
Leu	3.05	3.17	4.29	4.81	3.99	4.38	3.54	3.84	3.81	4.27
Orn	0.00	0.00	0.70	0.00	0.08	0.22	0.00	0.09	0.43	0.00
Lys	0.62	0.84	0.59	0.33	0.21	0.20	0.71	0.18	0.34	1.00
Pro	0.15	0.21	0.20	0.32	0.19	0.18	0.17	0.10	0.31	0.25
A/B ratio	0.79^{a}	1.16^{ab}	2.63^{ab}	1.83^{ab}	5.91^{b}	3.43^{ab}	1.08^{ab}	2.82^{ab}	1.92^{ab}	0.85^{ab}
TAA (mg /g shell)	79.71	88.93	63.82	53.85	81.23	65.92	78.17	81.95	137.61	74.98

Table 4. 9. List of fatty acid contents in nine different protein diets. Data are presented as means of three replicate samples. The nine diets were WF = White fish meal, RF = Red fish meal, BL = Blood meal, MB = Meat and bone meal, CA = Casein, SO = Soybean concentrate, GL = Gluten, MZ = Maize gluten and SP = *Spirulina*. The commercial diet was used prior to the start of the experiment. Superscripts represent significant differences (Dunn's tests; $p < 0.05$).

Fatty acid (mg / 100mg of fatty acids detected)		WF	RF	BL	MB	CA	SO	GL	MZ	SP	Commercial
Caproic acid	(C6:0)	0.83^{a}	0.58^{a}	2.56^{c}	1.71^{b}	1.61^{b}	0.79^{a}	0.62^{a}	0.63^{a}	0.45^{a}	0.53
Caprylic acid	(C8:0)	1.32^{a}	3.42^{bc}	4.27^{c}	2.88^{b}	2.10^{ab}	2.86^{b}	2.95^{b}	3.16^{bc}	1.12^{a}	1.07
Capric acid	(C10:0)	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.80^{b}	0.00
Undecanoic acid	(C11:0)	0.12^{ab}	0.10^{ab}	0.20^{b}	0.25^{bc}	0.40^{c}	0.09^{ab}	0.09^{ab}	0.08^{ab}	0.07^{a}	0.14
Tridecanoic acid	(C13:0)	0.26^{b}	0.45^{bc}	0.00^{a}	0.61^{c}	0.41^{bc}	0.10^{ab}	0.00^{a}	0.19^{ab}	0.24^{ab}	0.11
Myristic acid	(C14:0)	6.34^{ab}	8.76^{b}	13.72^{c}	4.82^{a}	10.15^{bc}	6.21^{ab}	5.79^{ab}	5.76^{ab}	5.13^{a}	2.81
Myristoleic acid	(C14:1)	0.59^{b}	0.34^{ab}	0.00^{a}	0.53^{b}	0.50^{b}	0.30^{ab}	0.33^{ab}	0.29^{ab}	0.26^{ab}	0.36
Penladecanoic acid	(C15:0)	0.00^{a}	0.00^{a}	0.00^{a}	1.34^{c}	0.66^{bc}	0.10^{ab}	0.39^{b}	0.39^{b}	0.23^{ab}	0.11
Palmitic acid	(C16:0)	15.74^{bc}	16.96^{bc}	20.71^{bc}	25.76^{c}	16.21^{bc}	12.86^{a}	14.76^{ab}	13.56^{ab}	27.27^{c}	13.90
Palmitoletic acid	(C16:1)	6.93^{bc}	7.44^{bc}	11.11^{c}	5.38^{ab}	7.46^{bc}	5.61^{ab}	5.22^{a}	5.05^{a}	5.13^{a}	2.09
Heptadecanoic acid	(C17:0)	0.62^{bc}	1.82^{c}	0.00^{a}	0.38^{ab}	0.57^{bc}	0.09^{a}	0.39^{ab}	0.09^{a}	0.11^{a}	0.24
cis-10-Heptadecanoic acid	(C17:1)	0.56^{b}	0.00^{a}	0.00^{a}	0.45^{b}	0.28^{ab}	0.48^{b}	0.00^{a}	0.25^{ab}	0.31^{ab}	0.08
Stearic acid	(C18:0)	3.39^{ab}	2.13^{a}	3.82^{ab}	4.64^{b}	3.33^{ab}	2.03^{a}	1.88^{a}	2.18^{a}	2.07^{a}	1.87
Elaidic acid	(C18:1n9t)	15.66^{bc}	4.96^{a}	12.37^{bc}	24.17^{c}	11.03^{b}	8.30^{ab}	7.73^{ab}	6.83^{ab}	6.76^{ab}	5.64
Oleic acid	(C18:1n9c)	7.79^{bc}	4.87^{ab}	6.93^{b}	10.24^{c}	5.13^{ab}	4.14^{a}	3.63^{a}	11.75^{c}	3.88^{a}	6.81
Linolelaidic acid	(C18:2n6t)	7.48^{a}	5.89^{a}	9.31^{ab}	9.65^{ab}	18.33^{b}	29.96^{c}	33.94^{c}	32.93^{c}	18.88^{b}	48.15
Linoleic acid	(C18:2n6c)	0.16^{a}	0.41^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.18^{a}	0.00^{a}	0.10^{a}	7.86^{b}	0.10
Arachidic acid	(C20:0)	2.70^{c}	1.41^{bc}	0.90^{ab}	1.33^{bc}	0.95^{ab}	0.37^{a}	0.20^{a}	1.21^{b}	0.18^{a}	0.14
r-Linolenic acid	(C18:3n6)	0.67^{b}	0.44^{ab}	0.00^{a}	0.47^{ab}	0.00^{a}	4.78^{a}	0.25^{ab}	2.11^{c}	0.42^{ab}	0.00
cis-11-Eicosenoic acid	(C20:1)	4.53^{bc}	2.49^{ab}	5.52^{c}	1.89^{a}	2.73^{ab}	2.55^{ab}	3.31^{b}	1.82^{a}	3.61^{bc}	2.00
Linolenic acid	(C18:3n3)	0.58^{ab}	2.97^{c}	0.00^{a}	0.32^{ab}	2.44^{bc}	2.27^{bc}	2.39^{bc}	1.39^{b}	1.20^{ab}	5.88
Haneicosanoic acid	(C21:0)	0.18^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.18^{b}	0.00
Behenic acid	(C22:0)	2.00^{bc}	1.01^{ab}	2.69^{c}	0.21^{a}	2.27^{bc}	1.14^{ab}	0.15^{a}	0.08^{a}	1.85^{b}	0.15
cis-8,11,14-Eicosadlenoic acid	(C20:3n6)	3.42^{c}	1.20^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	0.58^{ab}	0.89^{b}	0.00^{a}	0.62^{ab}	0.46
Erucic acid	(C22:1n9)	1.62^{bc}	0.00^{a}	0.00^{a}	0.53^{b}	0.21^{ab}	0.36^{ab}	3.33^{c}	3.30^{c}	0.31^{ab}	0.55
cis-11,14,17-Eicosatrienoic acid	(C20:3n3)	0.22^{b}	0.23^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.15^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00
Lignocaric acid	(C24;0)	2.25^{ab}	5.83^{c}	4.23^{bc}	0.83^{a}	3.93^{bc}	2.30^{ab}	3.46^{b}	1.26^{ab}	3.42^{b}	1.26
cis-5,8,11,14-Eicosapentaenoic acid	(C20:5n3)	8.61^{bc}	22.50^{c}	1.59^{a}	1.22^{a}	7.19^{bc}	8.45^{bc}	6.45^{bc}	4.30^{ab}	4.99^{ab}	3.56
Nervonic acid	(C24:1)	5.03^{d}	3.55^{c}	0.00^{a}	0.18^{ab}	1.98^{bc}	2.23^{bc}	1.68^{ab}	1.08^{ab}	1.54^{ab}	1.85
cis-4,7,10,13,16,19-Docosahexaenoic acid	(C22:6n3)	0.41^{bc}	0.23^{bc}	0.07^{a}	0.22^{b}	0.13^{ab}	0.73^{c}	0.16^{ab}	0.21^{b}	0.10^{ab}	0.14
Total fatty acids (mg / 100g tissue)		2752.41^{a}	3003.56^{a}	3195.59^{a}	4644.69^{a}	2938.08^{a}	4797.19^{a}	4593.39^{a}	4337.02^{a}	9659.35^{b}	2661.55
Total saturate fatty acid (%)		35.75^{ab}	42.48^{b}	53.09^{c}	44.76^{bc}	42.60^{b}	28.93^{a}	30.67^{a}	28.59^{a}	44.15^{bc}	22.32
Total unsaturated fatty acid (%)		64.25^{bc}	57.52^{b}	46.91^{a}	55.24^{ab}	57.40^{b}	71.07^{c}	69.33^{c}	71.41^{c}	55.85^{ab}	77.68
Total n-3 fatty acid (%)		9.82^{bc}	25.92^{c}	1.59^{a}	0.53^{a}	9.76^{bc}	11.60^{bc}	9.01^{b}	5.90^{ab}	6.28^{ab}	9.59
Total n-6 fatty acid (%)		11.72^{ab}	7.94^{a}	9.31^{ab}	10.13^{ab}	18.33^{b}	35.51^{c}	35.09^{c}	35.14^{c}	27.77^{bc}	48.71
n-3/n-6 ratio		0.84^{bc}	3.27^{c}	0.17^{a}	0.05^{a}	0.53^{b}	0.33^{ab}	0.26^{ab}	0.17^{a}	0.23^{ab}	0.20
Total n-9 fatty acid (%)		25.07^{cd}	9.83^{a}	19.30^{ab}	34.93^{a}	16.37^{bc}	12.80^{b}	14.69^{ab}	21.88^{c}	10.94^{ab}	13.00

Table 4. 10. List of fatty acid contents in soft bodies fed nine different protein diets. Data are presented as means of three replicate samples. The nine diets were WF = White fish meal, RF = Red fish meal, BL = Blood meal, MB = Meat and bone meal, CA = Casein, SO = Soybean concentrate, GL = Gluten, MZ = Maize gluten and SP = *Spirulina*. The commercial diet was used prior to the start of the experiment. N/A denotes not applicable. Superscripts represent significant differences (Dunn's tests; $p < 0.05$).

Fatty acid (mg / 100mg of fatty acids detected)		WF	RF	BL	MB	CA	SO	GL	MZ	SP	Commercial
Caproic acid	(C6:0)	0.71^{a}	5.52^{ab}	8.79^{b}	4.82^{ab}	0.70^{a}	0.85^{a}	14.55^{c}	2.09^{a}	4.81^{ab}	2.31
Caprylic acid	(C8:0)	0.43^{ab}	1.56^{ab}	0.00^{a}	3.07^{ab}	0.69^{ab}	0.35^{ab}	15.69^{c}	0.28^{ab}	11.57^{b}	4.39
Capric acid	(C10:0)	2.11^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00
Undecanoic acid	(C11:0)	0.23^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	0.13^{b}	0.08^{ab}	0.00^{a}	0.00^{a}	0.29^{c}	0.13
Tridecanoic acid	(C13:0)	0.30^{ab}	0.98^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.25^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.45
Myristic acid	(C14:0)	3.62^{a}	8.37^{bc}	11.85^{c}	4.59^{ab}	5.08^{b}	4.91^{ab}	7.26^{bc}	4.32^{ab}	6.67^{bc}	5.28
Myristoleic acid	(C14:1)	0.86^{bc}	0.86^{bc}	0.00^{a}	0.89^{bc}	0.92^{bc}	1.00^{bc}	1.42^{c}	1.28^{c}	0.49^{b}	1.35
Penladecanoic acid	(C15:0)	0.22^{ab}	0.00^{a}	0.00^{a}	2.39^{bc}	0.00^{a}	0.62^{b}	4.08^{c}	0.36^{ab}	0.00^{a}	0.34
cis-10-Pentadecenoic acid	(C15:1)	2.57^{b}	0.00^{a}	2.10^{ab}	0.00^{a}	1.19^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00
Palmitic acid	(C16:0)	11.24^{a}	28.80^{bc}	41.29^{c}	17.46^{ab}	13.04^{a}	20.92^{ab}	15.95^{ab}	23.44^{bc}	28.54^{ab}	22.45
Palmitoletic acid	(C16:1)	3.91^{bc}	1.06^{ab}	0.00^{a}	3.31^{b}	5.16^{c}	1.58^{ab}	0.00^{a}	1.27^{ab}	5.94^{c}	1.64
Heptadecanoic acid	(C17:0)	1.43^{b}	1.53^{b}	0.00^{a}	1.23^{b}	1.31^{b}	1.54^{b}	0.00^{a}	0.61^{ab}	0.53^{ab}	1.71
cis-10-Heptadecanoic acid	(C17:1)	1.37^{b}	0.00^{a}	0.00^{a}	0.65^{b}	1.05^{b}	0.69^{b}	0.00^{a}	0.94^{b}	0.00^{a}	1.10
Stearic acid	(C18:0)	5.08^{b}	7.65^{b}	15.02^{c}	7.52^{bc}	6.72^{bc}	7.55^{bc}	2.84^{ab}	6.47^{bc}	0.96^{a}	7.85
Elaidic acid	(C18:1n9t)	4.92^{ab}	6.65^{bc}	10.95^{c}	10.74^{c}	5.78^{b}	6.43^{bc}	1.51^{a}	5.28^{ab}	5.12^{ab}	5.54
Oleic acid	(C18:1n9c)	14.98^{bc}	14.35^{bc}	9.98^{b}	6.50^{ab}	16.31^{cd}	14.67^{bc}	19.60^{d}	10.62^{bc}	1.42^{a}	11.43
Linolelaidic acid	(C18:2n6t)	2.24^{ab}	0.00^{a}	0.00^{a}	4.90^{b}	2.21^{ab}	4.02^{ab}	2.74^{ab}	3.46^{ab}	15.75^{c}	4.16
Linoleic acid	(C18:2n6c)	0.20^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.25^{ab}	6.99^{b}	0.00
Arachidic acid	(C20:0)	1.48^{ab}	0.00^{a}	0.00^{a}	1.29^{ab}	2.49^{bc}	3.18^{c}	0.00^{a}	0.34^{ab}	0.66^{ab}	2.99
r-Linolenic acid	(C18:3n6)	0.75^{ab}	1.85^{c}	0.00^{a}	0.81^{b}	0.00^{a}	1.19^{bc}	0.00^{a}	0.94^{bc}	0.00^{a}	0.00
cis-11-Eicosenoic acid	(C20:1)	3.64^{c}	2.29^{b}	0.00^{a}	1.58^{ab}	1.53^{ab}	0.99^{ab}	0.00^{a}	2.50^{bc}	2.20^{bc}	0.71
Linolenic acid	(C18:3n3)	0.16^{ab}	0.00^{a}	0.00^{a}	1.11^{b}	0.71^{ab}	0.20^{ab}	0.00^{a}	0.39^{ab}	1.00^{b}	0.00
Haneicosanoic acid	(C21:0)	0.86^{ab}	1.20^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.24^{ab}	0.00^{a}	1.09^{b}	0.00^{a}	0.00
Behenic acid	(C22:0)	0.59^{ab}	0.51^{ab}	0.00^{a}	0.00^{a}	1.27^{b}	0.11^{ab}	2.25^{b}	0.34^{ab}	0.58^{ab}	0.55
cis-8,11,14-Eicosadlenoic acid	(C20:3n6)	6.57^{bc}	3.48^{ab}	0.00^{a}	12.57^{c}	9.60^{bc}	0.00^{a}	0.00^{a}	11.60^{c}	0.00^{a}	13.17
Erucic acid	(C22:1n9)	3.56^{c}	1.21^{ab}	0.00^{a}	0.00^{a}	0.47^{b}	2.26^{bc}	0.00^{a}	0.68^{ab}	0.89^{ab}	1.53
Lignocaric acid	(C24;0)	15.63^{c}	6.02^{ab}	0.00^{a}	6.28^{ab}	13.99^{bc}	16.07^{c}	7.34^{b}	13.12^{bc}	2.34^{ab}	6.65
cis-5,8,11,14-Eicosapentaenoic acid	(C20:5n3)	8.70^{b}	4.78^{ab}	0.00^{a}	7.41^{ab}	8.72^{bc}	9.61^{c}	2.53^{ab}	7.59^{ab}	2.30^{ab}	3.60
Nervonic acid	(C24:1)	1.03^{c}	0.39^{ab}	0.00^{a}	0.36^{ab}	0.63^{bc}	0.52^{b}	0.95^{bc}	0.32^{ab}	0.37^{ab}	0.27
cis-4,7,10,13,16,19-Docosahexaenoic acid	(C22:6n3)	0.61^{bc}	0.95^{c}	0.00^{a}	0.53^{abc}	0.28^{abc}	0.17^{ab}	1.30^{c}	0.40^{abc}	0.56^{abc}	0.39
Total fatty acids (mg / 100g tissue)		3167.84^{bc}	2250.33^{ab}	2097.44^{ab}	2377.13^{ab}	2843.44^{abc}	2669.71^{abc}	3276.20^{bc}	1801.78^{a}	3924.52^{c}	2439.25
Total saturate fatty acid (%)		43.93^{a}	62.15^{bc}	76.96^{c}	48.65^{ab}	45.43^{ab}	56.65^{bc}	69.96^{c}	52.47^{ab}	56.94^{bc}	55.11
Total unsaturated fatty acid (%)		56.07^{c}	37.85^{ab}	23.04^{a}	51.35^{bc}	54.57^{c}	43.35^{ab}	30.04^{ab}	47.53^{b}	43.06^{ab}	44.89
Total n-3 fatty acid (%)		9.47^{bc}	5.73^{ab}	0.00^{a}	9.05^{bc}	9.72^{bc}	9.98^{c}	3.83^{ab}	8.39^{b}	3.87^{ab}	3.99
Total n-6 fatty acid (%)		9.76^{b}	5.33^{ab}	0.00^{a}	18.28^{c}	11.81^{bc}	5.22^{ab}	2.74^{ab}	16.25^{bc}	22.74^{c}	17.33
n-3/n-6 ratio		0.97	1.07	N/A	0.50	0.82	1.91	1.40	0.52	0.17	0.23
Total n-9 fatty acid (%)		23.47^{c}	22.20^{bc}	20.94^{c}	17.24^{ab}	22.56^{bc}	23.36^{c}	21.11^{c}	16.57^{ab}	7.44^{a}	18.50

Chapter 5 Effect of dietary protein and temperature on the growth and health of New Zealand black-footed abalone (*Haliotis iris)* juveniles.

5.1 Abstract

The protein requirements for optimal growth and health of juvenile New Zealand black-footed abalone (*H. iris*) under different water temperatures were investigated. Six diets with different crude protein amounts (0, 10, 20, 30, 40 and 45%) and two temperature regimes (13-21°C, 8-16°C) were used to culture juvenile abalone over a four-month period. Growth (shell lengths, and animal weights) and health related (survival, activity, and mucous production) parameters were recorded for animals within all protein and temperature combinations. Proximate analyses and amino acid profiles also were performed on the diets, soft bodies (including gonad) and shells to evaluate the overall nutrient contents (diet and animal) and requirements (animal). Results indicate that protein requirements for juvenile *H. iris* are higher when environmental temperatures are low. Thus, increasing the dietary protein level results in better growth (i.e., increase in soft body [including gonad] crude protein and mean protein gain) and health (i.e., more goblet cells and thicker epitheial layers). Results from amino acid profiles in abalone soft bodies (including gonad) and shells show that these parameters did not change greatly with different dietary protein levels, but temperature did affect the concentration of several amino acids in both soft bodies (including gonad) and shells.

5.2 Introduction

Selecting the appropriate aquaculture feed is a biologically and economically important part of commercial cultivation. In some cases, up to 70% of the operating costs of aquaculture production may be associated with food production (Guillaume et al., 2001). Thus, it is critical to feed aquaculture stock balanced diets which meet nutritional requirements and maximize growth. Protein is one of the most important and expensive components in formulated diets for aquaculture purposes. Thus, attempts to optimize diets necessitate a thorough evaluation of the protein levels required (and essential amino acids) to efficiently improve growth of the target species. Dietary protein requirements are dependent

on a variety of factors, such as stock size, water temperature, feeding frequency, amount of non-protein dietary energy and dietary protein quality (Shimeno et al., 1980; Hidalgo et al., 1987). The combined effects of these factors are thought to determine the optimal dietary protein amounts and composition for fish and other aquatic invertebrates (Wilson, 1989).

Protein requirement studies have identified optimal protein levels for growth, body composition, and general health of a number of species, including a variety of molluscs (Uki et al., 1986c; Mai et al., 1995b; Coote et al., 2000; Hammer et al., 2006; Zhou et al., 2007). For example, an increase in the amount of protein (up to 45%) in the diet of juvenile ivory shells, *Babylonia areolate*, significantly increased soft body deposition compared to lower protein levels (Zhou et al., 2007). Comprehensive studies on the protein requirements for abalone are lacking, although recommended protein levels are listed for many species worldwide. These values include 20% for *H. discus* (Ogino and Kato, 1964), 20-32.3% for *H. discus hannai* (Uki et al., 1986c; Mai et al., 1995b), 23.3-35.6% for *H. tubercalata* (Mai et al., 1995b), 30% for *H. karntschatkana* (Taylor, 1997), 27% for *H. laevigata* (Coote et al., 2000), and 47% for *H. midue* (Britz, 1996a). However, these values mostly are based on qualitative measures, and often do not aim to achieve both optimal growth and reduced food costs. The broad range of these values among different abalone species also indicates that protein requirements are highly species-specific. In addition, protein requirements may change depending on the type of protein used. For example, Uki et al. (1986c) observed a maximum growth of *H. discus hunnai* when it was fed 43% protein from fish meal only, while about 23% protein was required to achieve the same growth when casein was used as the sole protein source. Protein requirements in abalone also may be affected by changes in carbohydrate and lipid contents in the diet (Kanuer *et al*., 1996; Tayler, 1997; Thongrod *et al.*, 2003).

Temperature has been identified as the most important abiotic process that affects growth in ectotherms, including aquatic invertebrates (Garcia-Esquivel et al., 2001, 2002; Moullac et al., 2007). It is one of the key factors governing the physiological processes of all organisms, and it directly affects food intake and metabolism (Hochachka and Somero, 1984; Jobling, 1994). In addition, temperature regulates protein turnover and growth efficiency (Jobling, 1994). Normally, animals exposed to optimal temperatures gradually increase growth with increasing food intake up to a maximum level (Tidwell et al., 2003). However, if the temperature increases above this optimal level, there is an immediate sharp decrease in

metabolic rate and food consumption, and growth inefficiency is observed (Brett, 1979; Jobling, 1994; Azevedo et al., 1998). Thus, for a given temperature, a differential in food intake and metabolic rate determines the energy available for growth (Brett and Groves, 1979; Jobling, 1994). Temperature also can affect digestibility of nutrients through its effect on food passage time and diffusion rates. Higher temperatures result in a reduced residence time in the gut, and less time for absorption, thus impinging on nutrient digestibility (McLeese and Stevens, 1982).

Most studies on the effect of temperature on protein requirements have been focused on fish, with a notable gap in regard to molluscan species. In fish, an increase in temperature may set off a gain in food intake and growth through stimulation of appetite (Austreng et al., 1987). Higher feeding frequency and activity may in turn increase the net uptake of nutrients. In abalone, the effect of temperature has been studied only in relation to food consumption (Britz et al., 1997; García-Esquivel et al., 2007), general growth (Leighton *et al.*, 1981; Steinarsson & Imsland, 2003) and temperature tolerance (Searle, 2004), and feed conversion ratio (García-Esquivel et al., 2007), but information regarding its effects on protein requirements is lacking. Furthermore, the New Zealand black-footed abalone (*Haliotis iris*) has received little attention with regard to its protein requirements and feeding preferences to maximize protein uptake and growth. Thus, the aim of this study was to determine the protein requirements for optimal growth and health of juvenile New Zealand black-footed abalone (*H. iris*) under different temperature conditions.

5.3 Materials and Methods

5.3.1 Experimental animals and culturing system

Juvenile *H. iris* were procured from Seahorses Australasia Limited, Warrington Dunedin, New Zealand. The animals were 2 to 2.5 years old from larval settlement, and their sizes ranged between 30 and 33 mm in maximum shell length. All individuals selected for the experiments were healthy and had clean shells. The animals were transported to the Portobello Marine Lab, University of Otago, Dunedin, New Zealand, were they were acclimatized in a flow-through tank for one month before the experiments was started. During the acclimation period, the animals were fed a commercial diet (Adam and Amos Abalone Foods, Australia). After the one-month period, the animals were carefully removed

from the holding tank with a flat, blunt blade. Then, the animals were dried with paper towels, and their initial maximum shell lengths (mm) and total wet weights (g) were recorded. Lengths were measured with vernier caliper (Mercer 0-125 mm) to the nearest 0.1 mm, and weights were measured with a digital balance (Mettler Toledo AB204-S) to the nearest 0.1 g. Five randomly selected animals were placed in each of 36 experimental containers (1 L in volume with square PVC lids, semi-transparent in color) with flow-through seawater. The containers were arranged into two temperature regimes (18 containers per temperature). The temperature regimes consisted of high (13 to 21 °C) and low (8 to 16 °C) temperatures, with a constant 5±0.3 °C difference between the two treatments throughout the experiment period (Fig. 5.1). Within each temperature treatment, the animals within three replicate containers were fed one of six diets, following a complete randomized design. The diets consisted of different amounts of protein (about 0, 10, 20, 30, 40, and 45%). The animals were then allowed to grow for four months. During this growth period, the water flowed through the containers at a rate of 500 ml/hour, which meant a total water exchange of 12 times per day. Water quality was always kept in good condition (pH between 8.3 to 8.5, dissolved oxygen > 10 ppm). Faeces and uneaten food residues were swept away every other day. The daily feeding rate was 5% of the total body weight per tank. An additional 36 animals were preserved (freeze dried or formalin) for analyses of initial body condition (see below).

5.3.2 Experimental diets

Six experimental diets were specifically formulated to contain different levels of protein, while trying to maintain all other components similar (Table 5.1). The aim was to formulate diets which contained 0, 10, 20, 30, 40, and 45% protein. While, these exact values were not possible to achieve, the final protein percents were similar to the target amounts, and will be referred to as 0, 10, 20, 30, 40, and 45% protein diets in this study. All dry ingredients were ground to 200 μm particle size, and then mixed in a blender without the starch and cellulose. Starch was then mixed with boiling water in order to activate the binding propensities (gelatinize). The activated starch and cellulose subsequently were added to the mixture of all other dry ingredients. The lipid oil was then added to form a dough. The dough was spread on a flat, square-sized board to 3 mm thickness. Small pieces (1 cm^2) were cut with a knife just prior to drying in a Mcgreger hot-air oven at 45 °C for about 12 hours, at which point the moisture level was reduced to less than 15%. The diets were then stored in a -20°C freezer

until they were used. Proximate analyses of these diets also were performed as indicated below.

5.3.3 Growth and health measurements

At the end of the four-month experiment, the maximum shell length and total wet weight of each animal were carefully measured, and survival rates were recorded. In addition, a flip test was performed on each animal to identify their activity level. For this test, the procedure was modified from Searle (2004) for approximating health of *H. iris* at the conclusion of the experimental trials. Specifically, the five animals from each container were placed upside down on a wet, flat surface. Then, the number of individuals that overturned to their normal position was recorded at 1 min intervals until all individuals had flipped to their normal position. After these tests, one of the five animals per container was used to obtain a histological section of the tentacle region, from which goblet cell counts and epithelium thickness measurements could be made. The remaining two to four animals per container (depending on mortality) were dissected to separate the shell, gonad and soft body (excluding gonad). Wet and dry weights and moisture contents were obtained for each of these three components. Then, the dried gonad and soft body (excluding gonad) of all the animals within each container were mixed together and ground up for proximate analyses and amino acid profiles.

5.3.4 Histological analyses

The animals for the histological analyses were first fixed in 10% buffered formalin for 48 hr. Each animal was imbedded in paraffin, and six 4 μm sections of the front tentacle area were cut with a rotary microtome (Leica RM2235). Three of these sections were stained with Haematoxylin and eosin to highlight the epithelial layer, and three sections were stained with Periodic Acid-Schiff (PAS) to highlight the tentacle mucus cells. Photographs of all sections were obtained under an Olympus BX41 light microscope. Within these thin sections, three randomly chosen 50 μm-length cross-sections of epithelial cell areas were used to count all goblet cells and to measure the epithelial thickness. The thickness of the epithelial layer was measured as the longest length of epithelial cross-section, which included the apical border. All measurements were performed with the NIH J program (provided by the Research Service

Branch of the National Institute of Mental Health, NIH).). The initial count of goblet cells and measurements of epithelial thicknesses were subtracted from the final values to standardize the data.

5.3.5 Proximate analyses

Proximate analyses were conducted on diet and soft body (including gonad) samples to obtain crude protein, crude lipid, ash and moisture contents following AOAC (1995). The Kjeldahl method was used for crude protein determination and the Bligh and Dyer (1959) method was used for crude lipid determination.

For crude protein extractions, about 0.5-1 g of dried diet and 0.2-0.3 g of dried abalone samples were used. These samples were digested in Velp tubes with 3 g of catalyst (9:1 mixture of potassium sulfate and copper sulfate) and 10 ml of concentrated sulfuric acid. The tubes with samples and blanks (no sample material) were placed in a Velp digester (Velp Scientifica Ltd.) within a hood. All samples were boiled for 3 hrs, and then diluted to 100 ml with mili-Q water. Five ml of each diluted digestion sample were distilled in a UDK126 semi-automatic steam distilling unit (Velp scientifica Ltd.). Then, the ammonia in these samples was collected in 250 ml Erlenmeryer glass flasks with 10 ml of 0.05N sulfuric acid and two drops of indicator, until a total volume of 150 ml was obtained. The indicator solution was made up by mixing 100 ml of 0.2% methyl red solution and 50 ml 0.1% methylene blue in ethanol. The amount of ammonia in each sample was obtained by titrating with a standardized 0.05 N sodium hydroxide solution. The percent of crude protein was calculated with the formula:

$$\text{Crude protein (\%)} = 0.0007 \times (Vb - Vs) \times (F \times 6.25 \times 20 / S) \times 100$$

Where, Vb = volume of standard 0.05N sodium hydroxide solution required for the blank (ml), Vs = volume of standard 0.05N sodium hydroxide solution in the sample, F = correction factor for the standard 0.05N sodium hydroxide solution in this study (0.4185), and S = sample weight (g).

The lipid extraction method was modified for small samples from Bligh and Dyer (1959). Dried diet (0.3 g) and abalone soft tissue (0.5 g) samples were hydrated in 0.7 ml distilled Milipore water and a 3ml mixture of methanol (2 ml) and chloroform (1 ml) in pre-weighed 25 ml screw top glass tubes. An ultrasonicater and nitrogen gas were used to enhance the extraction processes and prevent oxidation, respectively. While ultrasonicating, 1ml chloroform was added. After a 10 min interval, 1 ml of water was added, under nitrogen. The solution was then filtered with No.1 filter paper, and then centrifuged for 5 min at 2000 rpm. The lower organic solvent layer was collected into a pre-weighted test tube with a nitrogen stream gently blowing under a warm water bath until all the solvent was vaporized. Finally, the net weight of the lipids in the sample was recorded.

The amount of moisture in each diet and soft tissue sample was obtained from the net percent of water loss after freeze drying (Chist alpha series freeze dryer). The ash content was obtained by complete combustion in a 550 °C oven for 6 hrs.

The mean protein gain per abalone was calculated with the following equation:

MPG (mg/ abalone) = (SBt × 1-Mt × Pt) – (SBi × 1-Mi ×Pi) Mai et al. (1995b)

Where; SBt = final soft body weight (mg), Mt = moisture content (%), Pt = final protein content (%), SBi = initial soft body weight (mg), Mi = initial moisture content (%), and Pi = initial protein content (%).

5.3.6 Amino acid analyses

The amino acid analyses were conducted in accordance with Paramás et al. (2006). Three replicate 0.2 g dried powdered samples of abalone soft bodies were each mixed with 5 ml of water in 25 ml screw top glass tube. To each sample, a 5 ml 13 M concentrate hydrochloric with 0.1% phenol solution was added, and heated in a Velp digester at 110 °C for 12 hrs under nitrogen gas. After cooling, the solution was adjusted to a pH of 6.5-7.5 by adding 1 M NaOH solution. The final volume was recorded and stored in a -20 °C freezer until analysis. Samples were processed on a Shimadzu LC-10AD automatic sampling high-pressure liquid chromatography system (HPLC), fitted with a DGU-2A de-gas system with helium gas tank.

A mixed L-amino acids standard for these analyses was prepared in sodium phosphate buffer. This standard contained leucine (Leu) 1.844 g/L, isoleucine (Ile) 0.879 g/L, methionine (Met) 0.577 g/L, taurine (Tau) 0.189 g/L, tyrosine (Tyr) 0.125 g/L, glycine (Gly) 0.236 g/L, valine (Val) 0.925 g/L and Proline (Pro) from BDH; aspartic acid (Asp) 0.059 g/L, alanine (Ala) 0.007 g/L, and phenylalanine (Phe) 0.354 g/L from Merck; and γ-aminobutyric acid (GABA) 1.650 g/L, ornithine (Orn) 0.930 g/L, a-aminoadipic acid, histidine (His) 1.459 g/L, lysine (Lys) 0.361 g/L, glutamic acid (Glu) 0.272 g/L, threonine (Thr) 0.781 g/L, arginine (Arg) 0.381 g/L, serine (Ser) 0.395 g/L and 3,5-dibromotyrosine (I.S.) 1.785 g/L from Sigma. In addition, an internal standard (3, 5-dibromotyrosine) was added to the samples and the mixed L-amino acid standard. This internal standard (50 µl) was added providing a final concentration of $1x10^{-5}$ M. The samples were reacted with o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (MCE) at room temperature. The OPA regent was prepared by dissolving 500 mg of OPA (Merck) in 22.5 ml of ethanol. A total volume of 25 ml was obtained by adding 0.4M borate buffer (pH 10) and 400 µl of 2-mercaptoethanol (Sigma). The OPA regent was stored in a dark screw-top bottle at 4 °C. To avoid photo-oxidation, a fresh regent was prepared every three weeks. 50 µl of each sample was mixed with 450 µl sodium phosphate buffer (100 mM, pH 7.3; BDH) and 100 µl OPA regent and 50 µl of internal standard in a new 1.5 ml vial. These mixtures were prepared just prior to analyzing with HPLC. The reactions of the samples with OPA reagents yielded isoindolic derivatives to provide a fluorescence of analytes.

HPLC analyses were conducted with a solid phase consisting of a NOVA-PAK® C_{18} column (4 µm 3.9 × 150 mm Waters). The mobile phase was adapted from Paramás et al. (2006) to provide a gradient elution. The first mobile phase was 80:19:1 ratio of sodium phosphate buffer (10 mM, pH 7.3)/methanol/tetrahydrofurane (solution A). The second mobile phase contained a 20:80 sodium phosphate buffer (10 mM, pH 7.3)/methanol (solution B). Solution A was added at 100% at 0.1 ml/min for the first 3 min, after which time the flow rate was increased to 1.5 ml/min. At 14.5 min, the gradual addition of solution B was started. At 19.5 min, the ratio of solution A to B was 85:15. At 24.5 and 45 min, the ratios were 70:30 and 30:70, solution A to B, respectively. Fluorimetric detection of amino acids was obtained at excitation and emission wavelengths of 340 and 426 nm, respectively.

Because HPLC does not detect Proline, visible spectroscopy was employed to the same sample extracts prior to pre-treatment with OPA to detect this amino acid according to Amerine and Ough (1980). 0.1 ml of the previously hydrolyzed sample was transferred to a 15 ml screw-top test tube. To this sample, 5 ml of pure water, 0.25 ml formic acid, and 1 ml methyl cellosolve (2- methoxyethenol) ninhydrine solution (3 g of ninhydrine dissolved into 100 ml 2- methoxyethenol) was added and boiled with the cap for exactly 15 min in a water bath (Grant, SBB14). After cooling to room temperature, 1ml isopropanol-water mixture (1:1 by volume) was added. These solutions were transferred into cuvettes to measure absorbance with a spectrophotometer (Ultrospec 21000pro UV/Visible) at a 517 nm wavelength. The results were calculated on the basis of the curve formed by the standard solution.

Shell protein extraction followed methods by Mai et al. (2003). Individual shells were kept in 5% NaOH for 7 hrs, then rinsed thoroughly with deionized water, and air-dried for 24 hrs. 1 g powdered samples of newly-formed green shell (NS) and old brown shell (OS) were obtained and suspended in 5 ml of deionized water. These samples were passed through 3500 molecular weight cutoff dialysis membranes (Serva 44311 membra-cel ® dialysis tubing MW cutoff 3500 22 mm) for 72 hrs at room temperature against an external solution of 5% acetic acid and 0.01% (w/v) sodium azide. The acetic acid was removed from the sample remaining in the dialysis tubing with ultra-pure water for 48 hrs, and centrifuged at 10,000 g for 30 min at 4 °C. The resulting shell matrix protein (SMP) was collected from the precipitate by resuspending in 5 ml Millipore water for subsequent digestion and HPLC and proline analyses, as mentioned above.

5.3.7 Statistical analysis

Analyses of all growth and health parameters, except for survival and flip tests were conducted with two-way ANOVAs (diet and temperature as fixed factors) with Tukey *post-hoc* tests. For survival and flip test analyses, Kruskal-Wallis tests were performed with Dunn's multiple comparisons. Individual amino acids within different abalone components also were analyzed with Kruskal-Wallis tests and Dunn's multiple comparisons. Second order polynomial fittings were conducted to determine relationships between wet and dry soft body (including gonad) weights and protein gains against dietary protein levels under high

and low temperatures. All analyses were performed using a MINITAB version 14 software package.

5.4 Results

5.4.1 Abalone growth and health

Shell growth and total animal wet weight gains for juvenile *H. iris* maintained in experimental tanks for four months increased with increasing protein diets for both high and low temperature treatments (Fig. 5.2a, b). Shell growth ranged from 0.44±0.15 mm within 0% protein in the diet at high temperature to 4.74±0.24 mm with 30% protein in the diet at high temperature. The highest mean shell growth (±SD) of the diet and temperature combination (45% protein at high temperature) was 1.14±0.12 mm per month (38 μm day^{-1}). Wet weight gains ranged from -0.08± 0.19 g for animals fed low protein diet (~0%) to 2.64±0.64 g for animals fed diets with 40% protein, both in high temperature regimes. The diet and temperature combination which produced the highest mean (±SD) animal wet weight gain (40% protein at high temperature) was 0.63±0.08 g per month (21 mg day^{-1}). Two-way ANOVAs, with temperature and diet as fixed factors, revealed significant differences among diets (Table 5.2), and Tukey tests indicated distinct diet groupings (Fig. 5.2a, b). While high temperature treatments generally resulted in greater shell growth and animal wet weight gains, there were no statistical differences among 20-45% dietary protein treatments on mean shell growth, or among 30-45% protein treatments on mean wet weight gain (Table 5.2).

The survival of juvenile abalone after the four-month experimental periods was high, with mortalities observed only within 0 and 10% protein diets (Fig. 5.2c). A separate Kruskal- Wallis analysis was conducted for diet and temperature treatments, resulting in significant differences among diets, but no statistical differences were observed between temperatures (Table 5.3). A Dunn's tests indicated that only the 0% diet had significantly lower animal survival than the other diets (Fig. 5.2c).

The flip test results indicate that more time was required to flip abalone back to their normal position (recovery time) when the animals were fed 0 and 10% protein diets compared to those fed higher protein diets (Fig. 5.3a). The longest recovery period (3.93±0.23 min) was

observed for animals grown with 0% protein at high temperature, and the shortest recovery time was 1 min for animals fed 30-45% protein diets at both high and low temperatures. Results from separate Kruskal-Wallis analyses for diet and temperature indicate significant differences in the recovery time among diets, but no difference between temperatures (Table 5.3). A Dunn's tests indicated that only the 0% diet was significantly different from the others diets (Fig. 5.3a).

The change in goblet cell numbers and epithelial thicknesses were greater for juveniles maintained in experimental tanks with high protein diets for both high and low temperature treatments (Fig. 5.3b, c). However, the number of goblet cell decreased in animals fed 0 and 10% protein diets (for both temperatures), and the epithelial thickness decreased for animals fed 0% protein diets (for both temperature) and those fed 30% protein under low temperature (Fig. 5.3b, c). A separate set of animals were analyzed for their goblet cell counts and epithelial thickness at the start of the experiment. These animals had goblet cell counts of 19.7 cells per 50 μm epithelial length, and 5.6 μm in epithelial thickness. These values were subtracted for all results to highlight any changes (Fig. 5.3b, c). The adjusted final change in goblet cell counts ranged from -17.37±0.88 cells per 50 μm epithelial section for animals fed 0% protein at high temperature to 17.97±1.45 cells per 50 μm epithelial section for animals fed 40% protein at low temperature. The change in epithelial thicknesses ranged from -0.65±0.07 to 1.97±0.17 μm for animals fed 0% protein at high temperature and 45% protein at low temperature, respectively. Two-way ANOVAs, with temperature and diet as fixed factors, revealed significant differences between temperatures and among diets for both goblet cell counts and epithelial thickness analyses (Table 5.2). The interaction for the goblet cell count analysis was not significant, while the interaction for the epithelial thickness analysis was significant. Tukey tests indicated distinct diet groupings (Fig. 3b, c).

5.4.2 Proximate analyses

Proximate analysis results for the formulated diets indicated that the final diets generally were consistent with the initial targets for crude protein (Table 5.1). Crude lipid contents were difficult to maintain constant across diets, and they varied between 3.86±0.22 to 7.40±0.33%. However, there were no statistical differences among different diets, except for the 0% and 45% protein diets. Ash contents increased slightly with increasing protein content

diets, due to the greater amount of bone in the fish meal, which was used as a protein source. The moisture content was maintained relatively similar (13.41±0.31%) across all diets, although statistical differences were apparent among diets due to slight variations in fish meal and cellulose after the drying process.

The proximate analyses for abalone maintained within the various diets and temperature treatments for four months are shown in Figure 5.4 and Table 5.4. Crude protein content in the soft bodies (including gonad) for juvenile *H. iris* increased with increasing protein amounts in the diet for both high and low temperature treatments, but higher temperature yielded animals with greater protein contents (Fig. 5.4a). The percent crude protein content ranged from 55.99±1.03% within 0% protein in the diet at low temperature to 86.12± 2.47% with 40% protein in the diet at high temperature. Two-way ANOVAs, with temperature and diet as fixed factors, revealed significant differences among both diets and temperature, and interaction (Table 5.4). Tukey tests indicated paired diet differences (Fig. 5.4a).

Crude lipid contents in juvenile abalone differed among diets and temperature, but no clear trend could be observed (Fig.5.4b). However, all the animals had relatively low crude protein levels at the end of the experiment. Crude lipid contents ranged from 3.55±0.04% in animals within 45% protein in the diet at high temperature to 6.66±0.13% for animals fed 10% protein diets at high temperature. While significant differences were observed among diets and treatment interaction in a two-way ANOVA, temperature was not significantly different.

Significant differences were observed in the ash and moisture contents of animals grown under different diets and temperatures, but there were no clear trends in these treatments for either ash or moisture content analyses (Table 5.4; Fig. 5.4c). Ash contents ranged from 0.39±0.13% for animals fed 20% protein diets at low temperature to 1.97±0.08% for animals fed diets with 20% protein at high temperature. Moisture contents ranged from 78.89±0.45 to 84.99±0.34% for animals fed 20% protein at low temperature and 0% protein at high temperature, respectively.

5.4.3 Gonad and shell parameters

Both gonad wet and dry weights for juvenile abalone increased with increasing protein content in the diet at both high and low temperature treatments (Fig. 5.5a, b). However, no significant differences were observed between temperatures for either wet or dry gonad weights (Table 5.5). Gonad wet weights ranged from 0.30±0.02 g within 0% protein in the diet at high temperature to 0.92±0.04 g with 45% protein in the diet at low temperature. The mean gonad growth (±SD) with the best-performing diet and temperature combination was 0.23±0.01 g per month (45% protein at high temperature) for wet weight and 0.09±0.01 g per month (40% protein at high temperature) for dry weight.

Both shell wet and dry weights for abalone resulted in increasing trends with increasing protein content in the diet for both high and low temperature treatments (Fig. 5.5c, d). Significant differences also were observed between high and low temperatures, but not for the diet/temperature interaction (Table 5.5). Shell wet weights ranged from 1.44±0.11 to 2.39±0.17 g (10 to 40% protein range) for high temperatures, and 1.37±0.20 to 2.12±0.14 g (0 to 40% protein range) for low temperatures. Shell dry weights ranged from 1.37±0.11 to 2.22±0.16 g (10 to 40% protein range) for high temperatures, while dry weights ranged from 1.30±0.03 to 2.02±0.07 g (0 to 45% protein range) for low temperatures. The mean shell growth (±SD) of the best-performing diet and temperature combination (40% protein at high temperature) was 0.60±0.04 g per month wet weight and 0.56±0.04 g per month dry weight.

Results for wet and dry gonad to soft body (excluding gonad) ratios (GSI) resulted in no clear trends, although significant differences were observed for the temperature and interaction for wet GSI and for diet and interaction for dry GSI (Fig.5.6a, b; Table 5.6). Conversely, wet and dry soft body (including gonad) to shell ratios (SB/S) resulted in a slight increase in values from 0 to 10% protein diet treatments, which was maintained for higher protein content diets exposed to both temperatures (Fig. 6c,d). While significant differences were observed for both factors and interaction in the wet SB/S analysis, only diet was significant in the dry SB/S analysis (Table 5.6).

5.4.4 Protein and amino acid profiles

As expected, the total mean protein gain (MPG) for juvenile abalone resulted in increasing values with increasing protein in the diet for both temperatures (Fig. 5.7). Furthermore, negative MPG values were observed for animals exposed to 0 and 10% protein diets. MPG values ranged from -290.14±14.01 mg/abalone within 0% protein in the diet at high temperature to 215.83± 65.23 mg/abalone within 40% protein in the diet at low temperature. The mean daily protein per animal ranged from a loss of 2.42±0.11 mg/day, to a gain of 1.80±0.54 mg/day per for 0% protein at high temperature, to 40% protein at low temperature, respectively. A two-way ANOVA resulted in significant differences for diet, and non-significant differences for temperature and interaction (Table 5.6).

Second-order polynomial curves were fitted on three growth-related parameters (wet and dry soft body (including gonad) weights and mean protein gain) to estimate the protein requirements of juvenile abalone at high and low temperatures (Fig. 5.8). From these curves, R^2 values were obtained, ranging from 0.81 to 0.93 (Fig. 5.8, Table 5.7). The estimated mean protein requirement (X value at Y_{max}) differed between high and low temperature treatments. For high temperatures, the protein requirement ranged from 38.10 to 38.83% protein in the diet, and 42.25 to 53.75% for low temperature. A greater Y_{max} was found in low temperatures compared to high temperature treatments.

Amino acid compositions in the soft body (including gonad), and new and old shell material for abalone exposed to different diets and temperatures are shown in Tables 5.8-10. Separate Kruskal- Wallis analyses for different diets resulted in significant differences in alanine + taurine content for soft body (including gonad) analyses, and valine content for old shell material (Table 5.11). Temperature had a greater effect on amino acid composition for abalone (soft body [including gonad], and new and old shell material), as shown by the greater number of amino acids with significant Kruskal-Wallis results (Table 5.11). Individual Dunn's tests for paired diets (alanine + taurine and valine only) resulted in significantly different diet groupings, which reflect the increasing amino acid contents with increasing protein in the diets for soft body (including gonad) (alanine + taurine) and old shell material (valine).

5.5 Discussion

Results from the *H. iris* growth experiments under different diets and temperatures indicate that protein requirements for this species are affected by temperature. Thus, exposure of abalone to high temperatures results in lower protein requirements. However, temperature differences between 7 to 20°C have little effect on grow and health of *H. iris*. These findings suggest that if *H. iris* is grown within a low temperature range (8-16°C), a higher amount of protein (over 42%) will be needed to obtain optimal growth and health. In addition, increasing the dietary protein level results in better growth (i.e., increase in soft body (including gonad) crude protein content and mean protein gain) and health (more change in goblet cells and thicker epitheial layers). The results from the amino acid profiles in abalone soft bodies (including gonad) and shells show that these parameters did not change greatly with the different dietary protein levels, but temperature did affect the concentration of several amino acids in both soft bodies (including gonad) and shells. The specific effect of diet and temperature on various growth and health parameters is discussed below.

5.5.1 Growth – length and weight

Abalone growth measurements indicate that shell length growth and wet weight increased with increasing protein in the diet, under both high and low temperatures. Abalone achieved optimal growth (shell length and weigh) at around 40% of protein, and greater protein contents (> 40%) resulted in decreased growth. This general growth trend has been reported previously for other abalone species (Mai et al., 1995b; Britz, 1996a; Coote et al., 2000; Sales et al., 2003). For example, Sales et al. (2003) increased the protein level in the diet of *H. midue* from 27 to 47%, and observed an 18% weight gain. In addition, Mai et al. (1995b) fed juvenile *H. discus hannai* and *H. tuberculata* formulated diets with different protein amounts ranging from 0 to 50% protein, and found that diets containing 37 and 34% resulted in the highest weight gains, respectively. A similar decrease in growth was observed for these two abalone species when the protein amount was increased over the optimal levels (Mai et al., 1995b).

In this study, it was expected that the two temperature treatments would result in abalone growth differences. This expectation was based on Clarke (1998), who noted that abalone

from the North and South Island, New Zealand, should have different growths due to temperature differences (13-22 and 8-16 °C, respectively). While overall growth differences were not observed between the high (13-21 °C) and low (8-16 °C) temperature regimes, maximum growth was achieved at a lower dietary protein level for animals growing under high temperature. The implications of these findings are that abalone farms located in the north or south of New Zealand should attain the same stock growth by simply adjusting the protein level in the diet according to the temperature conditions. Previous studies on *H. iris* have demonstrated different optimal temperatures for growth of small (21-22 °C for 10 mm individuals) and large (20-21 °C for 30 mm individuals) animals fed the same quantity and quality of food (Seale, 2004). Based on these values, the experimental animals of this study (33 mm in shell length) were grown under optimal temperatures within the high temperature regime. However, animals within the low temperature regime (8-16 °C) achieve similar growths, but only at a higher protein level (40%).

5.5.2 Health and survival

Abalone survival within all diets and temperature regimes was 100%, except for those exposed to 0 and 10% protein, indicating that the experimental setup provided good conditions for these animals. In addition, these results suggest that a basic 15% protein diet is necessary for good survival for this species. Few studies have investigated the effect of low protein level diets on abalone species. However, in a similar study, Mai et al. (1995b) found that over 96% survival rates could be achieved for juveniles *H. discus hannai* and *H. tuberculata* on 10% or more protein after 100 days.

The results for the flip test were consistent with those for abalone survival, where longer recovery times (poorer health) were observed for animals grown with under 10% protein diets, and shorter recovery times (good health) were recorded for animals fed over 15% protein. The flip test was first employed by Searle (2004) to evaluate general vitality and health of juvenile *H. iris* grown under different temperature regimes. Searle (2004) found that exposure to higher temperatures resulted in longer recovery periods and higher mortality rates, and he related this test with generally poor health. In the present study, longer recovery periods also were associated with lower number of globet cells and thinner epithelial thickness in the tentacles, which results in less mucous production and stickiness, and lower

ability to support the flip movement. The tentacles normally are the first areas to make contact with the surface while attempting to flip back to normal position. Animals with poorer health tend to produce less mucous, and less stickiness may result in a lesser ability to overturn. Thus, these results suggest that the number of globet cells and epithelial thickness provide a good index for mucous production, ability to flip back to normal position, and is a good external indicator of abalone health.

Abalone grown in the low temperature regimes had a significantly greater number of globet cells and thicker epithelial layers than those grown in high temperature regimes. Previous studies have shown that gastropods move less during winter (colder temperatures) and produce more high protein mucous to increase attachment strength (Donovan, 1998; Smith and Morin 2002). In agreement with this report, this study showed that animals fed higher protein diets also had significantly more globet cells and thicker epithelial layers. It is likely that the production of adhesive mucous is energetically costly for this abalone species. Indeed, Smith and Morin (2002) showed that mucous production in resting limpets (*Lottia limatula*) contained 2.7 times more protein that the mucous produced during high activity periods.

5.5.3 Proximate analyses

Results from the proximate analyses of abalone grown with different protein diets closely reflected their diet compositions. For example, animals fed higher protein diets contained higher crude proteins at the end of the experiment and *vice versa*. Similar results have been observed for other abalone species, including *H. discus hannai* and *H. tuberculata* (Mai *et al.*, 1995b) *H. midae* (Britz, 1996), *H. laevigata* (Coote *et al.*, 2000), and *H. fulgen* (Gómez-Montes *et al*., 2003). In addition, no clear trend was observed in this study for crude lipids, ash content or moisture, indicating that these parameters were not affected by the differences in protein content among diets. Similar observations were reported by Gómez-Montes *et al*. (2003), who found that a variability of 26-41% dietary crude protein did not affect the soft body ash or moisture contents of *H. fulgen* juveniles. While the present study did not show a lower lipid content in animals fed high dietary protein, previous studies have shown this trend for other species, including other abalone species (Mai *et al.*, 1995a) and ivory shell snails (Zhou *et al.*, 2007). Furthermore, Durazo-Beltrán *et al.*, (2004) found that juvenile green

abalone (*H. fulgens*) increased their soft body lipid contents when they were starved (no protein intake). Such body composition differences among species may be related to variations in the metabolic rates of food components, such as proteins and carbohydrates. However, investigations on these metabolic processes are lacking presently, and future studies would need to incorporate isoenergetic and isolipidic experiments to elucidate these species-specific differences.

Significantly larger moisture contents were observed in animals kept at high temperature regardless of dietary protein. Similar observations were reported by García-Esquivel *et al*. (2007) on *H. fulgens* juveniles. He found that abalone reared at 20°C had significantly lower moisture in their soft bodies and higher soft body / shell ratios compared to animals reared at 25°C. These differences were attributed to better feed conversion ratios and protein efficiency ratios for animals in low temperature treatments compared to those in high temperature treatments. In the present study, significant temperature effects were only found in soft body / shell ratios when wet weights were used in the calculation, but not for calculations based on dry weights. Thus, it is suggested that abalone may improve their dietary protein absorption under lower temperatures, which may affect their osmoregulation, and thus increase their soft body weight. Indeed, higher water contents in liver and muscle tissues also were recorded with increasing acclimation temperatures in goldfish (Hoar & Cottle, 1952; Kanungo & Prosser, 1959).

5.5.4 Gonad and shell parameters

While increasing gonad weights were observed with increasing protein in the diet of *H. iris*, the gonad to soft body (excluding gonad) index (GSI) did not change. This trend indicates that gonad weight increased with the same magnitude as the increase in body weight. Similarly, shell weights were higher in animals fed higher protein levels. In contrast, the soft body (including gonad) to shell weight ratio (SB/B) stayed constant across different protein levels, but only for diets containing 10-45% protein. Animals fed no protein had a significantly lower SB/B ratio. Mai et al. (1995b) also found that there was no significant difference in SB/S ratios when juvenile *H. discus hannai* and *H. tubercalata* were fed diets containing 20-50% protein levels. However, Britz (1996a) found that the condition factor (an analogous measure of SB/S) was lower when juvenile abalone (*H. midae*) were fed 24%

dietary protein compared to those fed 34 and 44% protein. Such variations in SB/B suggest species-specific differences which may need further investigation.

5.5.5 Optimum dietary protein level and temperature requirements for growth

The abalone feeding experiments indicate that within both temperature regimes, a 40% protein diet generally provides the best performance for the growth and health indicators tested. Second-order polynomial regression curves for body weight and protein gain also showed best performance within a range of 38-54% dietary protein, depending on the temperature regime and the biological parameter used. This type of regression curve is commonly used to identify nutrient and protein requirements for aquaculture species, such as the abalone *H. discus hannai* and *H. tuberculata* (Mai et al., 1995b) and *H. laevigata* (Coote et al., 2000). In such studies, weight gains were used to identify daily growth and to estimate protein requirements (Zhou et al., 2007; Singh et al., 2009). In the present study, the regression curves suggest that animals exposed to higher temperatures would be expected to have lower protein requirements (about 38%), and those exposed to lower temperatures would be expected to have higher protein requirements (about 42-54%, depending on sample water content). Investigations on the effect of temperature on abalone protein requirements are lacking. Instead, previous studies report protein requirement values for one temperature only (Mai et al., 1995b; Coote et al., 2000). For example, *H. discus hannai* and *H. tuberculata* require 35.6 and 32.3% protein, respectively, at 13-15 °C (Mai et al., 1995b), and *H. laevigata* requires 27% protein at 20 °C (Coote et al., 2000), based on this type of regression curves. With this information, it appears that *H. iris* requires a higher amount of protein in its diet when exposed to either low or high temperatures. One potential reason for this discrepancy is that the studies by Mai et al. (1995b) and Coote et al. (2000) utilized casein and gelatin as protein sources, which may be easier for abalone to digest. In contrast, in this study, *H. iris* juveniles were fed a red fish meal protein source, which may be more difficult for this species to digest. Thus, digestibility variations among species and culturing conditions also may be responsible for these protein requirement differences.

Although growth generally increases with increasing dietary protein levels, the utilization efficiency depends on the rate of protein conversion into new body tissues. Beyond the maximum protein requirement, any protein surplus will cause a reduction in growth efficiency

(Elliot, 1976). Past this maximum point, there is an increase in catabolism, production of ammonia, and inefficient protein utilization (McCallum and Higgs, 1989; Hewitt, 1992; Singh et al., 2008, 2009). For *H. iris*, a drop in body weight from 0.66 to 0.63 is estimated with a 10% increase in dietary protein past the maximum of 40% at high temperature, and a drop of 0.59 to 0.57 g with a 10% increase over the 45% maximum requirement at low temperature. The decrease in body growth and protein gain for this species under low temperature reflects a change in metabolic rate. A similar decrease in metabolic rate and protein efficiency are observed in oysters as a response to lower temperatures (Moullac et al., 2007). This physiological response is not observed in all poikilotherms (Hidalgo and Alliot, 1988; Singh et al., 2008, 2009). For example, in juvenile seabass (*Dicentrarchus labrax*) the protein requirement does not change across a temperature range of 15 and 20 °C. Instead, they increase their food consumption to compensate for the changes in metabolic rate caused by temperature (Hidalgo and Alliot, 1988). In Atlantic salmon, the same protein efficiency is maintained under different temperatures with a trypsin isozyme variant enzyme, which is more active at low temperatures (Rungruangsak-Torrissen et al., 1998). Other fish species may dampen changes in body temperature by moving to habitats with different temperatures (Kelsh and Neill, 1990), and thus sustaining stable protein efficiency.

5.5.6 Amino acid profiles

There were little differences in amino acid profiles for soft body (including gonad) and shell material with changes in dietary protein amounts. However, alanine + taurine contents were higher when animals were fed 45% protein compared to 0%. This trend reflects the fact that starving animals will use alanine + taurine as a food source. Viana et al. (2007) described how *H. fulgens* starved for 27 days experienced a significant reduction in alanine, proline, tyrosine, serine, and glutamic acid in the plasma, indicating the utilization of these amino acids to maintain energy levels. Significant differences in alanine + taurine contents in the soft body (including gonad) between temperatures indicate an accumulation of these amino acids in the body of abalone exposed to low temperatures. Low temperatures may decrease both the metabolic rate and secretion of digestive enzymes which, in turn, cause a decrease in protein efficiency. A similar decrease in metabolic rate was observed in *H. kamtschatkana* from summer to winter (Donovan, 1998). In addition, a decrease in alanine + taurine was reported for the oyster *Crassostrea gigas*, with increasing temperature, reflecting a higher metabolic rate (Moullac et al., 2007).

The overall implications of this work are that protein requirements for juvenile *H. iris* are higher when environmental temperatures are low. Thus, an increase in dietary protein can be used as a cheaper alternative to increasing water temperature in culturing tanks during winter or in the South Island, New Zealand. In addition, changes in dietary protein do not affect the amino acid profiles in young abalone. Conversely, animals cultured at different temperatures do experience specific changes in the amino acid composition of soft body (including gonad) and shell materials. The implications of these compositional changes will need further investigation. Future studies also should be focused on the relative digestibility and the metabolism of feeds with varying amounts of protein

Figure 5. 1. Daily records of water temperature for the high and low temperature treatments during the four-month experimental period (1 March to 30 June, 2004).

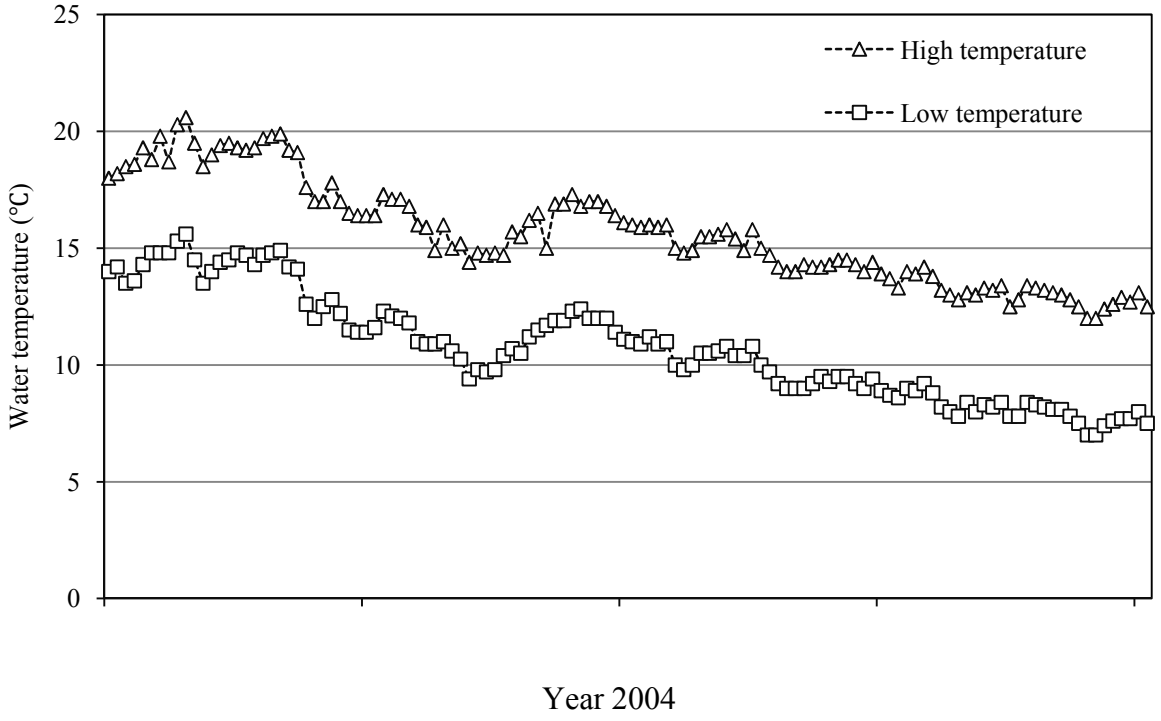


Figure 5. 2. (a) Maximun shell length growth, (b) total animal wet weight gain, and (c) survival, after the four-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%). Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$) for shell length growth and weight gain data analyses, and Dunn's test ($p < 0.05$) for survival data analyses.

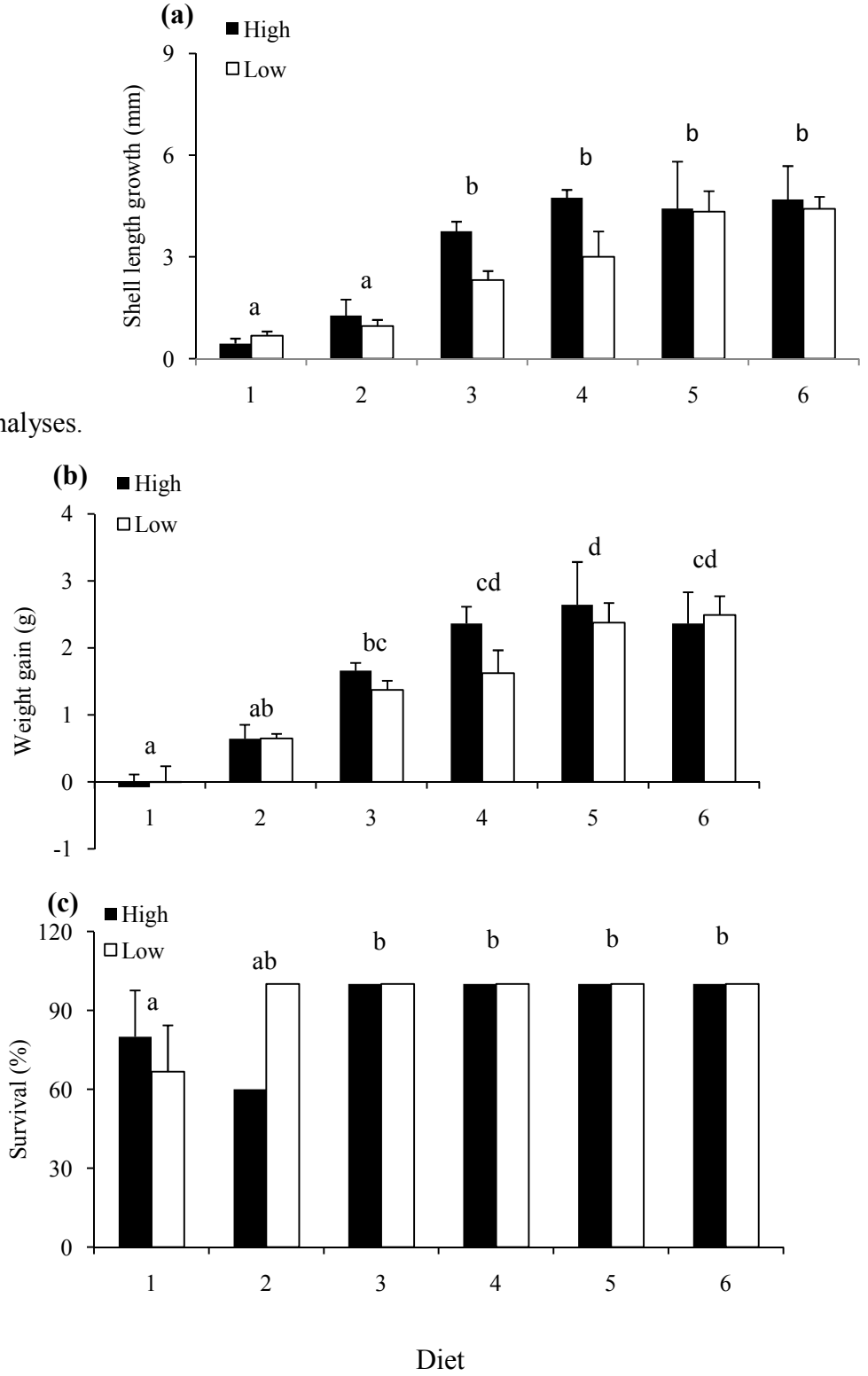


Figure 5. 3. (a) Flip time, (b) change in goblet cell number, and (c) change in epithelial thickness after the four-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%). Different letters on top of the error bars denote significant differences resulting from Dunn's test ($p < 0.05$) for flip time and Tukey *post-hoc* tests ($p < 0.05$) for goblet cell number, epithelial thickness.

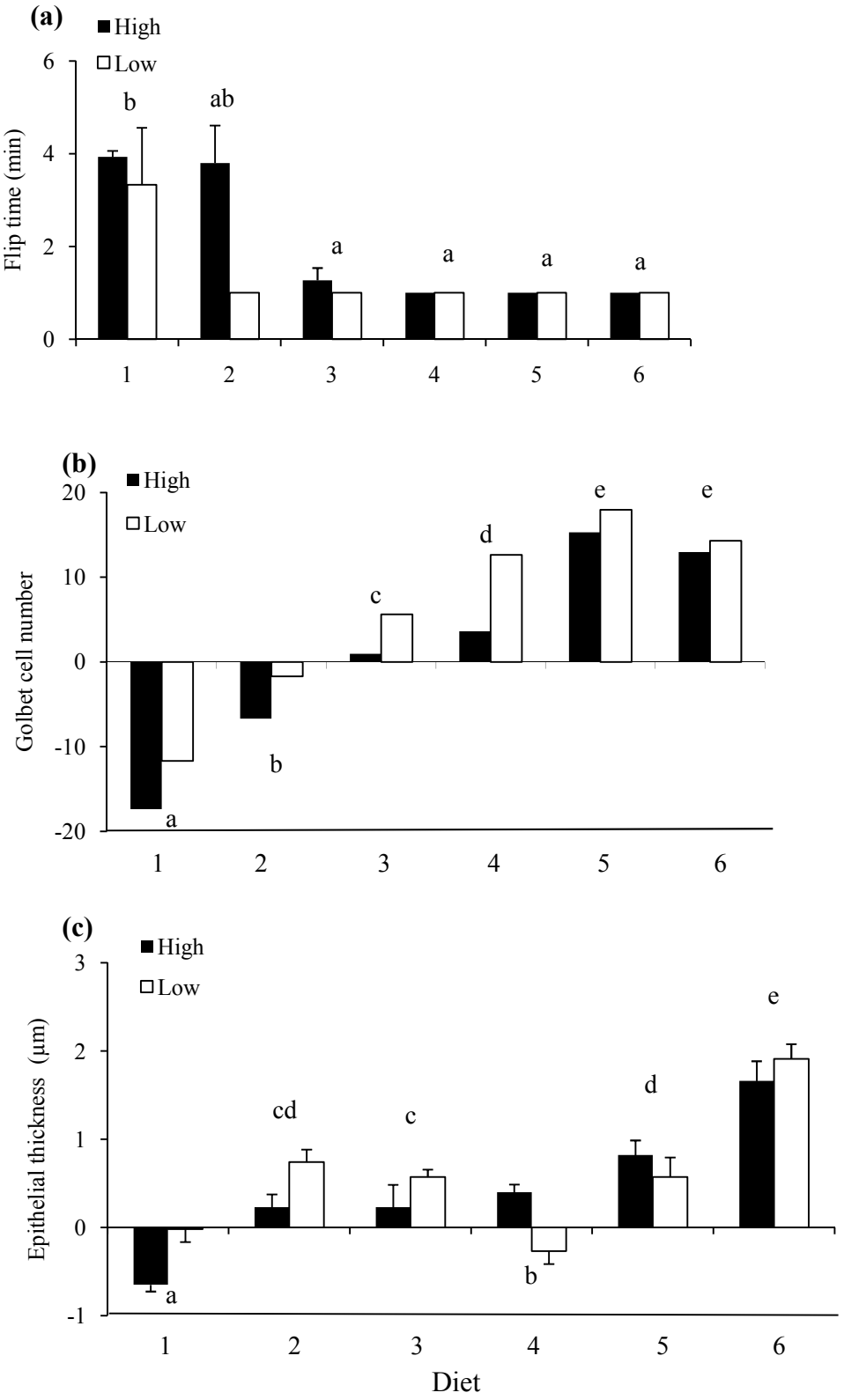


Figure 5. 4. Proximate analysis of soft bodies (including gonad) of experimental animals, including (a) crude protein, (b) crude lipid, (c) ash, and (d) moisture after the four-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%). Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

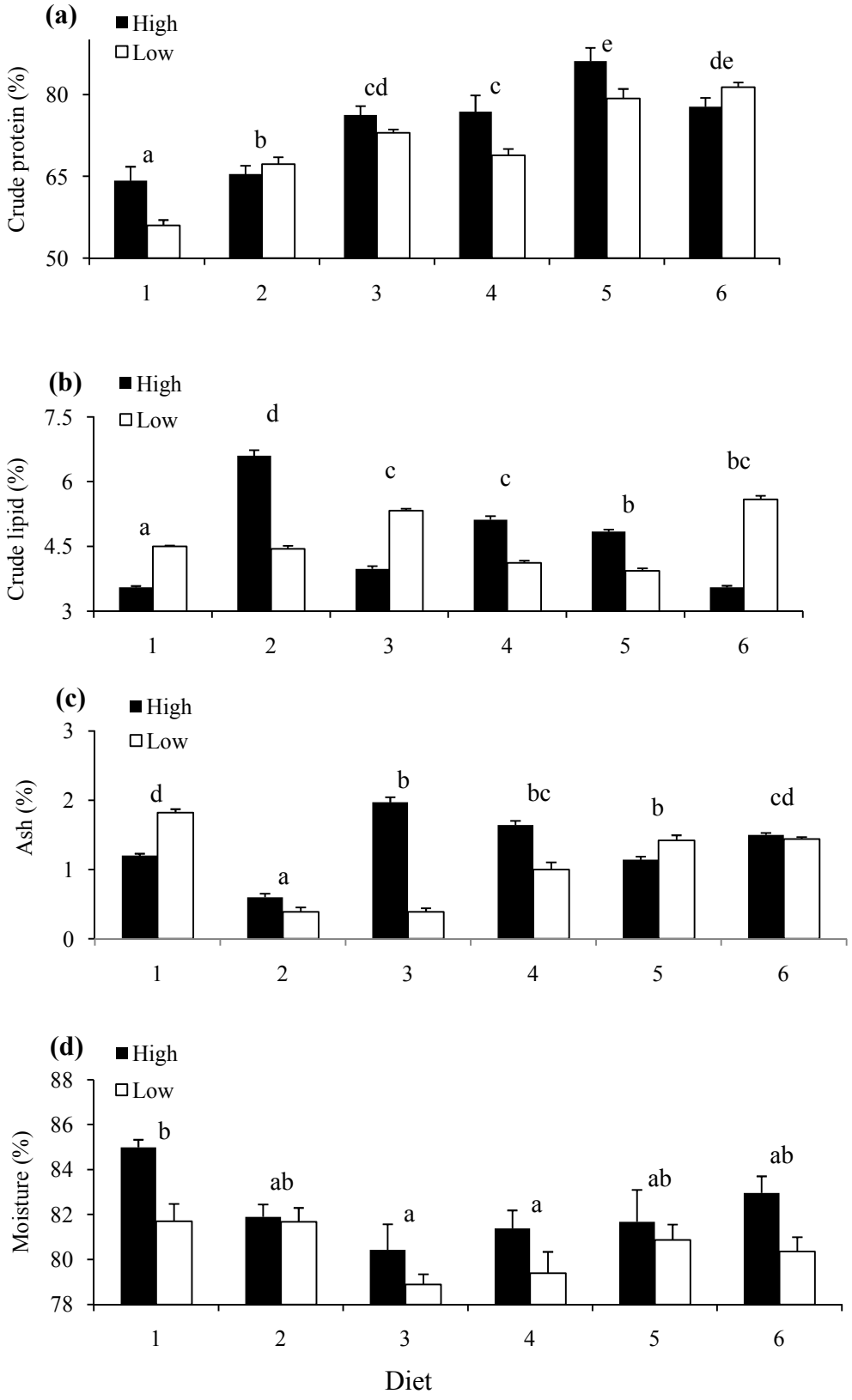


Figure 5. 5. Gonad (a) wet and (b) dry weights, and shell (c) wet and (d) dry weights of animals after the four-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%). Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

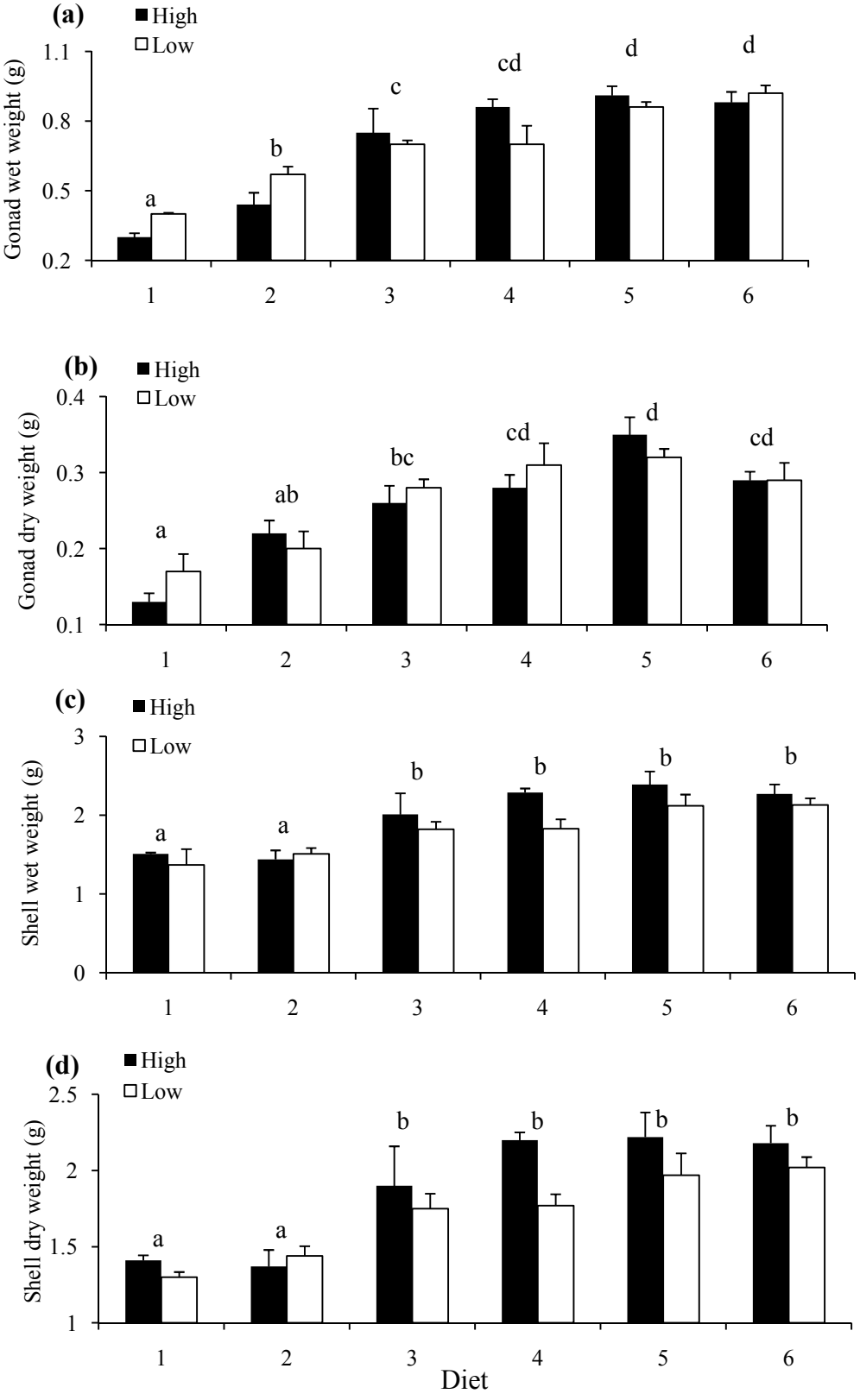


Figure 5. 6. (a) wet and (b) dry gonad soft body (excluding gonad) indices, and (c) wet and (d) dry soft body (including gonad) shell ratios (SB/S) of animals after the four-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%). Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$)**.**

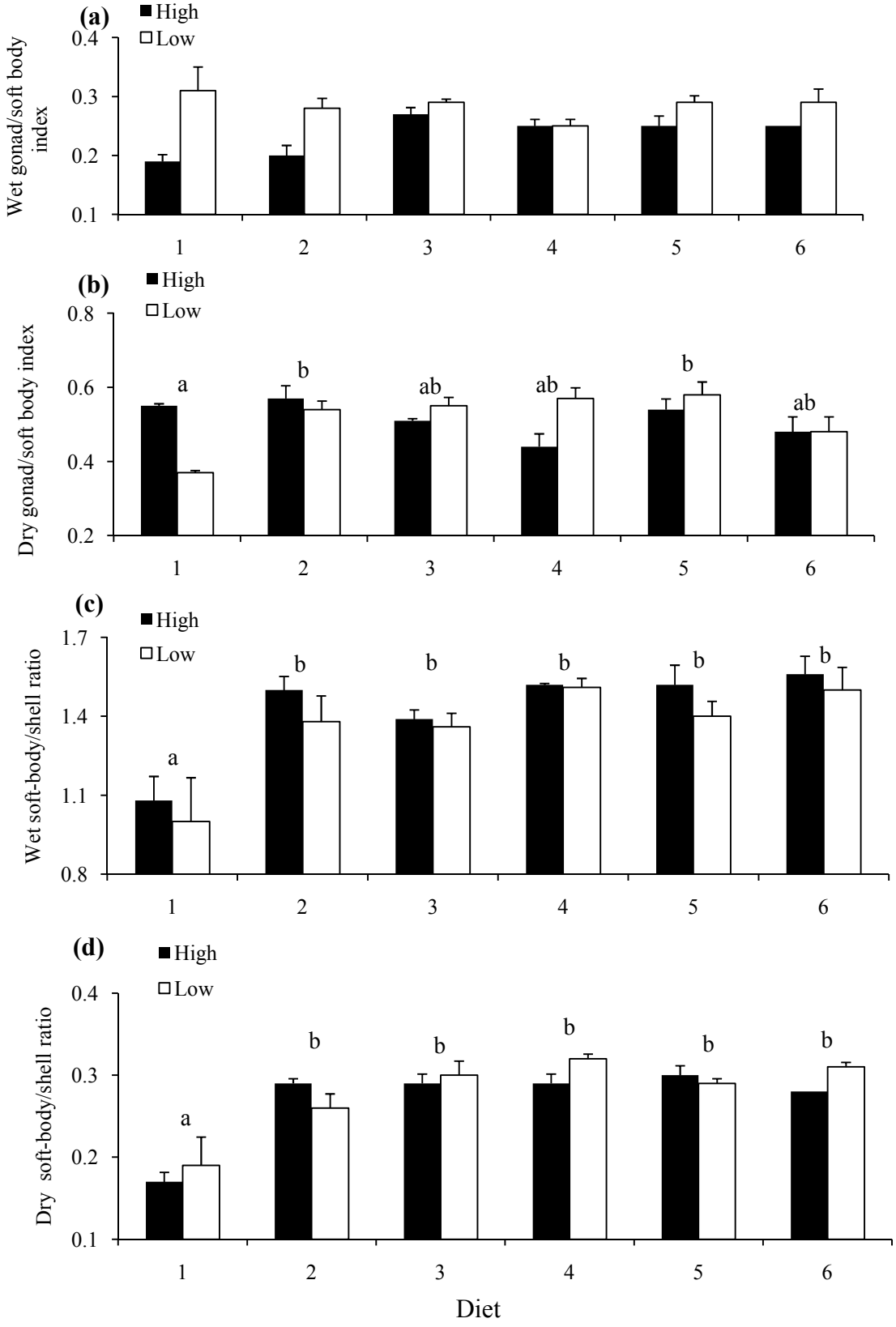


Figure 5. 7. Mean protein gain (MPG) after the four-month experimental period. Data are presented as means ± SD of three replicate experimental tanks. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%). Different letters on top of the error bars denote significant differences resulting fromTukey *post-hoc* tests ($p < 0.05$).

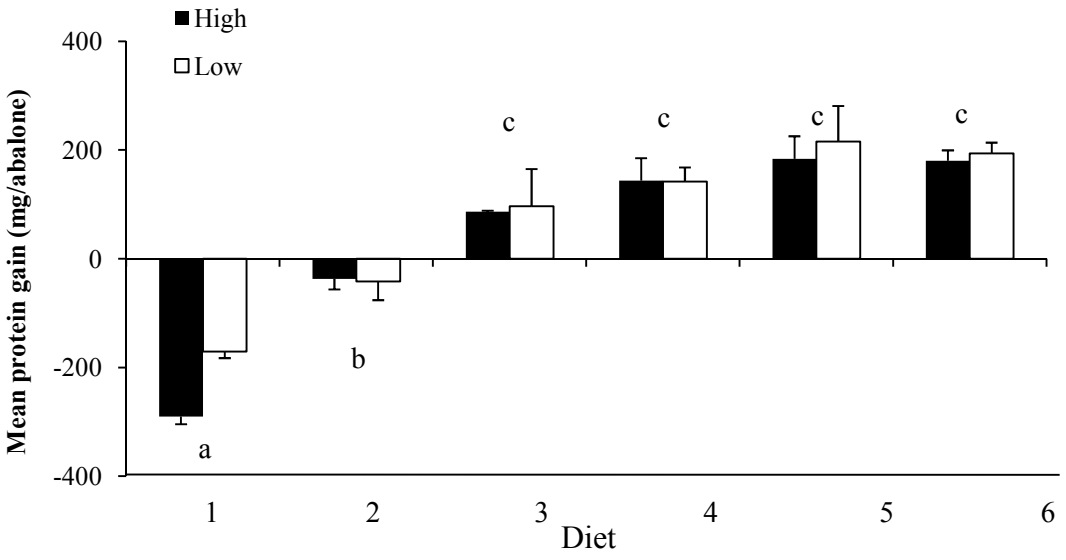


Figure 5. 8. Second-order polynomial curves obtained from (a) dry soft body (including gonad) weight and (b) mean protein gain against dietary protein level.

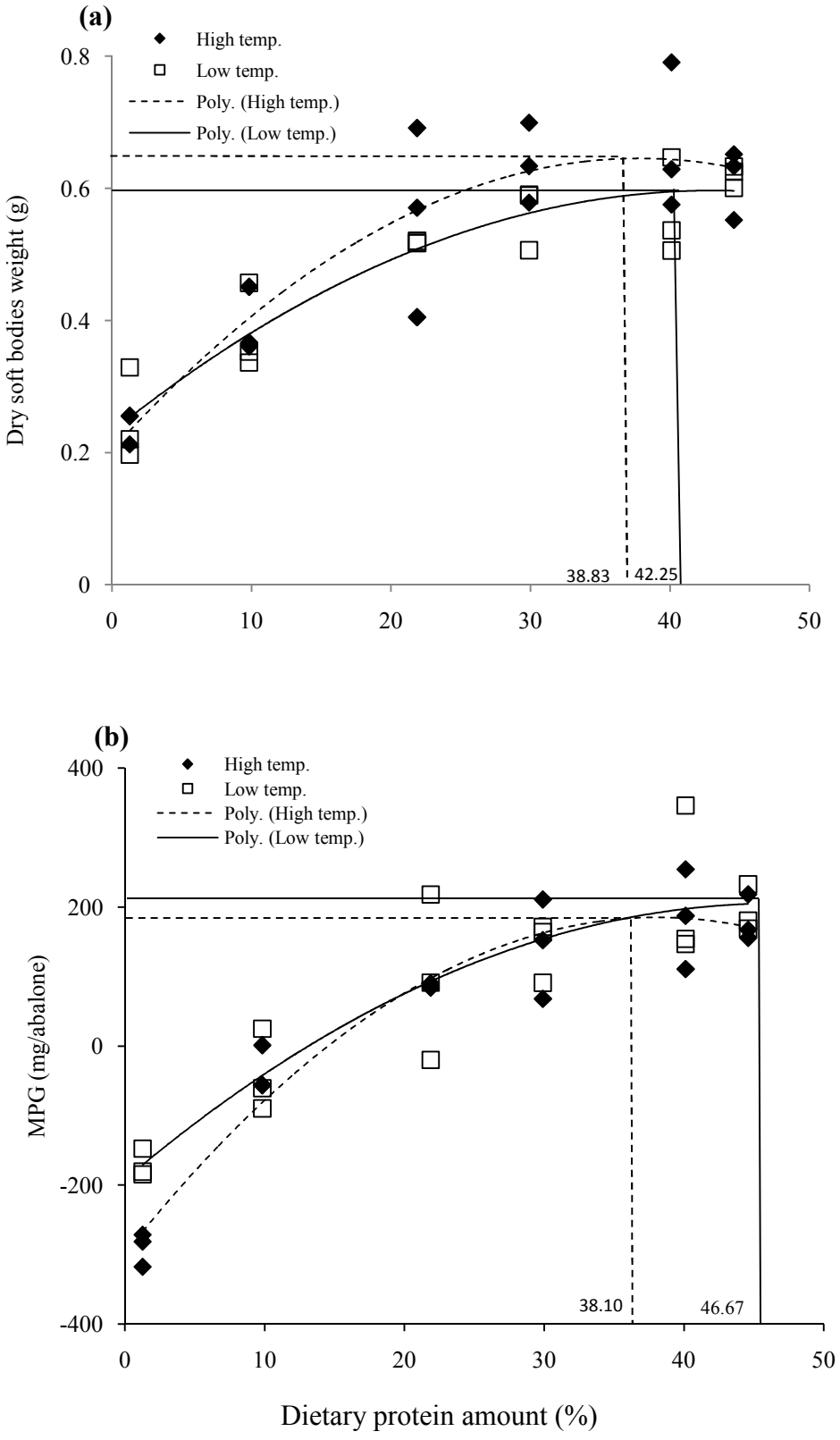


Table 5. 1. Diet recipes and proximate analyses of diets. Data are presented as means ± SD of three replicate samples. Six diets were formulated with different protein amounts (1= 0%, 2= 10%, 3= 20%, 4= 30%, 5= 40%, and 6= 45%). The crude energy for 100 g of diet was calculated from the sum of mean gross energy per gram of carbohydrate (maintained constant at 40% in the diet with 4 kcal energy per gram), lipid (9 kcal) and protein (4 kcal) in each diet (Brett and Grove, 1979). Capital superscript letters indicate significant differences (Turkey $p < 0.05$).

Diet number	*1*	*2*	*3*	*4*	*5*	6
Protein amount	0%	10%	20%	30%	40%	45%
Red fish meal[a]	-	9.6	19.6	29.6	39.6	44.6
Cellose[b]	49.8	41.5	32.8	24.1	15.4	11.7
Starch[c]	40.0	40.0	40.0	40.0	40.0	40.0
Lipid[d]	7.2	5.9	4.6	3.3	2.0	0.7
Vitamine[e]	1.5	1.5	1.5	1.5	1.5	1.5
Minerial Mix[f]	1.5	1.5	1.5	1.5	1.5	1.5
Proximate analyses						
Crude protein (%)	1.27±1.51	9.83±0.95	21.87±0.93	29.90±4.25	40.11±1.22	44.58±2.57
Crude lipid (%)	7.40 ± 0.33^{C}	3.89 ± 0.12^{A}	3.86 ± 0.22^{A}	5.12 ± 0.35^{AB}	4.23 ± 0.33^{A}	5.76 ± 0.37^{B}
Ash content (%)	3.43 ± 0.003^{A}	8.34 ± 0.030^{B}	11.83 ± 0.007^{C}	16.34 ± 0.021^{D}	20.91 ± 0.004^{E}	21.26 ± 0.003^{F}
Moisture (%)	13.81 ± 0.008^{F}	13.19 ± 0.002^{B}	13.57 ± 0.001^{D}	12.96 ± 0.001^{A}	13.68 ± 0.001^{E}	13.23 ± 0.001^{C}
Crude energy (Kcal/100g diet)	231.68	234.33	276.06	325.68	358.51	390.16
Average pellet weight (g)	0.19	0.22	0.23	0.25	0.28	0.31

a. red fish meal supplied by Sealord, Nelson (NZ) Ltd..

b. from commercial product "Just-Fiber ™"

c. modified starch (Firm-MIX ™) used for binding pellets from New Zealand Starch Ltd.

d. cold-filter cod liver oil (Healther™, NZ).

e. Vitamin mix (Shin- Chan™, Taiwan) per g contain: Riboflavin 10mg, PABA 40mg, Pyridoxine HCl 4mg, Niacin 80mg, Ca pantothenate 20mg, Inositol 400mg, Ascorbic acid 400mg, Biotin 1.2mg, Vitamin E 45mg, Menadione 8mg, Vitamin B_{12} 18mg, Vitamin A 10000 I.U., Vitamin D 200 I.U., Ethoxyquin 40mg, Folic acid 3mg, Thiamin HCl 12mg.

f. Mineral mixture g/kg mixture: NaCl (BDH) 10g, $MgSO_4 \cdot 7H_2O$ (BDH) 10g, $NaH_2PO_4 \cdot 2H_2O$ (BDH) 150g, KH_2PO_4 (BDH) 250g, K_2HPO_4 (BDH) 320g, $Ca(H_2P0_4)_2 \cdot H_2O$ (Merck) 200g, Fe-lactate (SIGMA) 25g, Ca-lactate (SIGMA) 35g, $ZnS04 \cdot 7H_2O$ (BDH) 3.53g, $MnS0_4 \cdot 4\ H_2O$ (BDH) 1.62g, $CuSO_4 \cdot 5H_2O$(BDH) 310mg, $CoCl_2 \cdot 6H_2O$ (Merck) 10mg, KIO_3 (Merck) 30mg.

Table 5. 2. Two-way ANOVA results for growth and health parameters.

	Maximum shell length growth				*Total animal wet weight gain*			
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.76	0.79	0.380	1	0.29	1.03	0.320
Diet	5	10.98	11.39	0.001	5	6.28	21.96	0.001
Interaction	5	0.69	0.71	0.620	5	0.16	0.55	0.730
Error	24	0.96			24	0.29		
Total	35				35			
	Change in goblet cell count				*Change in epithelial thickness*			
Source	df	MS	F	p	df	MS	F	p
Temperature	1	200.65	36.65	0.001	1	0.16	6.62	0.020
Diet	5	816.76	149.20	0.001	5	3.10	125.11	0.001
Interaction	5	10.50	1.92	0.130	5	0.37	14.97	0.001
Error	24	5.47			24	0.03		
Total	35				35			

Table 5. 3. Kruskal-Wallis test results for survival and flip tests.

Survival

Diet as fixed factor					Temperature as fixed factor				
	N	Median	Rank	Z		N	Median	Rank	Z
0%	6	70.00	10.00	-2.16	High	18	100.00	17.10	-0.78
10%	6	90.00	13.00	-1.40					
20%	6	100.00	22.00	0.89	Low	18	100.00	19.90	0.78
30%	6	100.00	22.00	0.89					
40%	6	100.00	22.00	0.89					
45%	6	100.00	22.00	0.89					
Overall	36		18.50		Overall	36		18.5	
H = 17.18 df = 5 p = 0.004 (adjusted for ties)					H = 1.26 df = 1 p = 0.262 (adjusted for ties)				

Flip test

Diet as fixed factor					Temperature as fixed factor				
	N	Median	Rank	Z		N	Median	Rank	Z
0%	6	3.82	31.70	3.35	High	18	1.00	20.60	1.22
10%	6	1.70	22.80	1.08					
20%	6	1.00	16.10	-0.62	Low	18	1.00	16.40	-1.22
30%	6	1.00	13.50	-1.27					
40%	6	1.00	13.50	-1.27					
45%	6	1.00	13.50	-1.27					
Overall	36		18.5		Overall	36		18.5	
H = 20.91 df = 5 p = 0.001 (adjusted for ties)					H = 2.11 df = 1 p = 0.147 (adjusted for ties)				

Table 5. 4. Two-way ANOVA results for proximate analyses on soft body (including gonad) of abalone juveniles.

	Crude protein				*Crude lipid*		
Source	df	MS	F	p	df	MS	F	p
Temperature	1	110.36	11.58	0.001	1	0.02	1.27	0.270
Diet	5	419.25	44.00	0.001	5	1.47	102.53	0.001
Interaction	5	39.48	4.14	0.010	5	4.01	280.29	0.001
Error	24	9.53			24	0.01		
Total	35				35			

	Ash content				*Moisture content*		
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.63	58.98	0.001	1	27.35	14.00	0.001
Diet	5	0.82	76.90	0.001	5	9.67	4.95	0.001
Interaction	5	0.90	83.85	0.001	5	1.92	0.98	0.450
Error	24	0.01			24	1.95		
Total	35				35			

Table 5. 5. Two-way ANOVA results for growth parameters.

	Gonad wet weight				*Gonad dry weight*			
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.00	0.00	0.950	1	0.00	0.35	0.560
Diet	5	0.29	40.54	0.001	5	0.03	23.24	0.001
Interaction	5	0.02	2.48	0.060	5	0.00	1.02	0.430
Error	24	0.01			24	0.00		
Total	35				35			

	Shell wet weight				*Shell dry weight*			
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.32	5.51	0.030	1	0.27	6.28	0.020
Diet	5	0.76	13.12	0.001	5	0.68	16.06	0.001
Interaction	5	0.05	0.79	0.570	5	0.04	0.96	0.460
Error	24	0.06			24	0.04		
Total	35				35			

Table 5. 6. Two-way ANOVA results for gonad soft body (excluding gonad) index (GSI), soft body (including gonad) shell ratio (SB/S) and mean protein gain.

	GSI wet				*GSI dry*		
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.02	23.89	0.001	1	0.00	0.00	1.000
Diet	5	0.00	1.53	0.220	5	0.01	4.06	0.010
Interaction	5	0.00	2.99	0.030	5	0.02	6.61	0.001
Error	24	0.00			24	0.00		
Total	35				35			

	SB/S wet				*SB/S dry*		
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.50	26.78	0.001	1	0.00	1.01	0.320
Diet	5	0.47	24.98	0.001	5	0.01	21.64	0.001
Interaction	5	0.23	12.17	0.001	5	0.00	1.40	0.260
Error	24	0.02			24	0.00		
Total	35				35			

	Mean protein gain			
Source	df	MS	F	p
Temperature	1	7121	1.81	0.190
Diet	5	165482	42.11	0.001
Interaction	5	3238	0.82	0.550
Error	24	3930		
Total	35			

Table 5. 7. Second-order polynomial fittings obtained from different parameters against dietary protein amount.

Parameter	*Temperature*	*Equation*	*R square*	*X value at* Y_{max}	Y_{max}
Wet soft body weight	High	$Y=-0.002X^2+0.15X+0.21$	0.87	38.30	3.02 g
Wet soft body weight	Low	$Y= -0.0008X^2+0.09X+1.45$	0.81	53.75	3.98 g
Dry soft body weight	High	$Y=-0.0003X^2+0.02X+0.20$	0.82	38.83	0.52 g
Dry soft body weight	Low	$Y= -0.0002X^2+0.02X+0.23$	0.88	42.25	0.71 g
Protein gain	High	$Y= -0.34X^2+25.40X-298.7$	0.93	38.10	175.49 mg/abalone
Protein gain	Low	$Y= -0.18X^2+17.03X-192.1$	0.83	46.67	210.63 mg/abalone

Table 5. 8. Amino acid composition of soft bodies (including gonad) (units: mg/100mg of total amino acids detected). Data are presented as means ± SD of three replicate experimental samples. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%).

	High temperature						*Low temperature*					
Diet	1	2	3	4	5	6	1	2	3	4	5	6
Asx	7.64	6.30	5.34	5.65	5.87	6.32	6.37	4.80	5.75	6.22	5.62	5.14
Glx	9.38	7.87	7.90	7.48	7.35	9.03	8.14	7.19	7.42	8.28	9.20	8.31
Ser	15.30	14.04	12.85	14.96	15.70	14.92	16.56	16.09	15.32	15.32	14.42	14.16
His	1.50	1.32	1.50	1.75	1.58	1.70	1.49	1.49	1.58	1.50	1.54	1.73
Gly	8.92	7.41	7.27	7.97	7.92	7.69	6.76	7.57	7.35	6.79	8.22	6.25
Thr	6.87	6.74	6.44	6.76	8.07	7.44	8.62	8.41	8.00	8.54	7.56	8.21
Arg	7.89	6.96	6.39	7.15	5.89	6.07	6.85	6.80	6.66	6.16	6.52	5.70
Ala + Tau	13.24	13.50	14.40	16.45	15.21	16.01	14.25	16.26	16.27	15.78	15.81	17.68
GABA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tyr	1.40	1.36	1.54	1.66	1.70	1.69	1.72	1.64	1.60	1.74	1.60	1.75
Met	1.25	1.44	1.10	1.60	1.74	1.46	1.57	1.64	1.70	1.38	0.97	1.33
Val	2.38	2.66	2.54	2.45	3.02	2.81	3.58	3.36	3.05	3.50	3.14	3.63
Phe	4.35	4.91	5.68	4.95	5.41	5.16	4.83	4.59	5.10	5.04	4.74	4.87
Ile	1.82	2.34	2.75	1.77	2.38	2.18	2.64	2.57	2.32	2.59	2.46	2.80
Leu	7.45	9.76	12.00	8.00	8.66	8.31	8.33	8.54	8.59	8.85	8.24	9.49
Orn	1.16	2.78	1.56	0.94	0.68	0.48	0.35	0.16	0.46	0.39	0.42	0.60
Lys	2.31	4.34	4.94	4.01	3.47	3.20	1.76	2.74	2.81	2.26	3.54	3.17
Pro	0.32	0.23	0.29	0.24	0.26	0.30	0.28	0.25	0.25	0.34	0.38	0.26

Table 5. 9. Amino acid composition of new shell material (units: mg/100mg of total amino acids detected). Data are presented as means ± SD of three replicate experimental samples. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%).

	High temperature						*Low temperature*					
Diet	1	2	3	4	5	6	1	2	3	4	5	6
Asx	8.93	14.29	11.25	13.13	19.73	11.60	11.97	14.05	7.77	12.50	11.05	13.08
Glx	8.18	8.73	7.42	7.02	8.64	11.88	14.24	8.58	9.97	12.92	11.84	14.10
Ser	20.39	22.12	19.60	19.08	19.76	20.74	19.35	22.79	16.51	19.55	19.77	20.20
His	15.32	11.28	15.52	17.24	10.97	15.36	10.53	14.63	18.33	16.89	19.75	11.12
Gly	4.14	2.62	3.65	3.53	2.93	1.65	2.05	1.31	1.18	2.11	3.41	2.30
Thr	6.77	4.71	8.00	7.41	3.75	7.76	2.36	8.67	5.58	5.17	1.59	3.57
Arg	2.42	2.00	2.25	2.44	2.26	2.90	2.30	2.38	2.38	2.55	2.72	2.69
Ala + Tau	14.25	13.30	12.99	12.96	12.04	10.24	6.20	8.69	9.38	9.47	9.87	11.22
GABA	3.49	3.43	3.66	3.05	2.39	2.81	2.79	2.35	2.04	2.61	2.99	1.67
Tyr	1.61	2.60	1.62	1.23	2.03	1.56	1.24	1.65	2.10	1.57	1.54	2.24
Met	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Val	2.82	2.90	2.47	2.19	2.79	2.39	3.34	2.69	3.65	2.88	3.12	3.81
Phe	5.22	5.23	5.06	4.56	5.91	5.08	6.91	5.79	4.55	5.66	5.69	6.40
Ile	1.07	1.20	1.13	0.74	1.00	0.92	1.39	1.13	0.84	1.20	1.26	1.41
Leu	4.39	4.43	4.11	3.72	4.69	4.06	5.36	4.26	14.76	4.02	4.28	5.01
Orn	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lys	0.63	0.66	0.77	0.92	0.58	0.62	0.46	0.54	0.57	0.64	0.79	0.39
Pro	0.37	0.52	0.51	0.79	0.53	0.43	0.49	0.49	0.38	0.28	0.32	0.79

Table 5. 10. Amino acid composition in old shell material (units: mg/100mg of total amino acids detected). Data are presented as means ± SD of three replicate experimental samples. Six diets were formulated with different protein amounts (1 = 0%, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, and 6 = 45%).

	High temperature						*Low temperature*					
Diet	1	2	3	4	5	6	1	2	3	4	5	6
Asx	10.62	12.16	11.98	11.94	12.62	14.47	11.13	8.94	8.96	9.74	11.11	9.36
Glx	8.97	7.14	9.31	10.20	10.48	12.51	14.81	13.42	12.77	14.20	16.65	13.04
Ser	20.70	19.40	20.67	19.98	20.73	22.99	20.66	19.80	19.74	20.19	21.24	20.37
His	14.62	18.07	13.23	9.13	9.67	9.92	12.12	16.48	19.08	15.40	5.76	13.96
Gly	3.75	4.17	3.01	2.35	2.60	1.47	1.69	1.06	1.05	1.90	1.88	2.60
Thr	5.46	6.05	6.48	7.19	6.03	3.28	6.17	5.82	4.98	4.07	5.55	3.76
Arg	2.06	1.93	2.22	2.21	2.19	2.34	2.75	2.67	2.51	2.38	2.42	2.87
Ala + Tau	12.88	12.13	12.11	12.85	12.26	8.22	9.43	10.75	10.61	9.74	8.50	9.21
GABA	2.80	2.86	2.94	2.80	2.82	1.77	1.68	2.12	2.04	1.90	1.79	2.59
Tyr	2.39	1.95	2.72	3.74	3.72	5.00	2.70	3.34	3.28	3.70	4.70	3.49
Met	0.19	0.20	0.08	0.12	0.18	0.37	0.20	0.46	0.22	0.28	0.22	0.30
Val	2.88	2.44	3.03	3.77	3.49	5.40	2.94	3.03	2.91	3.82	4.90	4.03
Phe	5.03	4.67	5.21	5.91	5.93	5.70	6.02	5.64	5.32	5.48	6.63	6.45
Ile	1.11	1.01	1.05	1.10	1.07	1.06	1.15	1.01	1.01	1.26	1.64	1.44
Leu	4.63	3.97	4.73	5.56	4.97	4.50	4.73	4.37	4.26	4.56	5.41	5.04
Orn	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lys	0.80	1.20	0.62	0.67	0.62	0.21	0.62	0.45	0.47	0.65	0.51	0.40
Pro	1.12	0.67	0.60	0.47	0.63	0.78	1.22	0.64	0.81	0.75	1.07	1.09

Table 5. 11. Kruskal-Wallis test (n=3; dietary protein level and temperature as fixed factors) on individual amino acids in soft body (including gonad) and both new and old shell material. Bold numbers indicate significant differences ($p < 0.05$). N/A indicates values lower than the detection limit.

	Analyses by diet protein amount			*Analyses by temperature*		
	Soft body	New shell	Old shell	Soft body	New shell	Old shell
Asx	0.327	0.254	0.935	0.248	0.954	**0.004**
Glx	0.712	0.691	0.628	0.729	**0.007**	**0.000**
Ser	0.493	0.264	0.348	0.248	0.686	0.773
His	0.517	0.620	0.394	0.564	0.386	0.453
Gly	0.860	0.549	0.876	0.204	**0.013**	**0.013**
Thr	0.958	0.210	0.769	**0.001**	0.133	0.248
Arg	0.299	0.997	0.423	0.729	0.001	**0.011**
Ala + Tau	**0.044**	0.334	0.880	**0.033**	0.184	**0.001**
GABA	N/A	0.848	0.989	N/A	**0.033**	**0.003**
Tyr	0.475	0.472	0.160	0.119	0.386	0.488
Met	0.965	N/A	0.218	0.525	N/A	0.133
Val	0.735	0.806	**0.015**	0.001	0.106	0.644
Phe	0.272	0.527	0.399	0.356	0.094	**0.043**
Ile	0.832	0.718	0.271	0.083	0.094	0.083
Leu	0.222	0.595	0.195	0.686	0.817	0.729
Orn	0.995	N/A	N/A	**0.005**	N/A	N/A
Lys	0.337	0.738	0.337	0.057	0.386	0.488
Pro	0.928	0.852	0.487	0.525	0.166	0.133

Dunn's test results ($p < 0.05$): 0% < 45% for Ala + Tau in soft body (including gonad) and 10% < 45% for Val in old shell material.

Chapter 6 Effects of dietary protein source and amount in shell morphology of juvenile abalone (*Haliotis iris*).

6.1 Abstract

The aim of this study is to investigate the effect of dietary nutritional value (protein source and amount) and temperature on shell morphology of cultured abalone juveniles. Morphological variations have long been used by ecologists and evolutionary biologists to identify physiological and/or environmental conditions associated with the growth and health of organisms. Two important factors affecting morphological plasticity are food supply and temperature conditions. Two different experiments were conducted to test the effect of dietary protein (source and amount) and water temperature on shell morphology (length, width, height, thickness, and weight) of juvenile abalone (*Haliotis iris*). In the first experiment, nine different dietary protein sources (white fish and red fish meals, blood meal, meat and bone meal, casein, soybean concentrate, wheat gluten, maize gluten and *Spirulina* powder) resulted in wider, higher, and heavier shells with casein diets, and flatter shells with red fish meal. Abalone fed blood meal produced significantly narrower, thinner, and lighter shells compared to animals fed the other diets. In the second experiment, six different dietary protein amounts (0, 10, 20, 30, 40, and 45%) and two temperature regimes (13-21 °C and 8-16 °C) resulted in wider and heavier shells with increasing protein content. However, shell height and thickness were not affected by different protein amounts. Significant differences between temperatures were observed only for shell height and thickness. The results suggest that diet and temperature may be used to manipulate abalone shell morphology in aquaculture environments, and they may be potential factors in shell variations of wild populations.

6.2 Introduction

Morphological variations provide an important index for understanding the distribution of species and diversification processes. Thus, an entire field of eco-morphology has been dedicated to investigating the relationships between an organism's morphology and the environmental conditions and changes over different time scales (Wainwright and Reilly, 1994). A number of studies have illustrated how specific marine environmental conditions result in animals having distinct morphological characteristics (Lively, 1986; Shadrin and

Lezhnev, 1990; Saunders et al., 2008). For example, Lively (1986) reported that the barnacle (*Chthamalus anisopoma*) may grow with different morphologies depending on the level of predation at a given site. Other studies have identified plastic responses (short temporal scales) to local environmental stressors (Lively, 1986; Johannesson et al., 1990; Robles and Robb, 1993; Trussel, 1996; Stefani and Branch, 2003). For instance, the shell height and width of *M. galloprovincialis* has been shown to vary by about 4-9% depending on the level of sewage contamination in the environment (Shadrin and Lezhnev, 1990). Furthermore, juvenile trochus snails (*Trochus niloticus* L.) are known to change their shell morphology (smooth *versus* rough) depending on the environmental conditions (wild *versus* hatchery) (Chunhabundit et al. 2001). Within longer temporal scales, morphological variability has been associated with genetic differences resulting from historical selective pressures for a particular morphotype (Etter, 1996; Luttikhuizen et al., 2003; Swain et al., 2005).

Abalone have been shown to have high morphological variability among populations inhabiting different coastal sites (Worthington et al., 1995; Prince et al., 2008; Saunders et al., 2008). It has been suggested that morphological variation in abalone is a result of plastic response to food availability, such as drift algae (Day and Fleming, 1992; Shepherd and Steinberg, 1992; McShane and Naylor, 1995; Saunders et al., 2009). Shepherd and Hearn (1983) reported that *H. rubra* have rounder shells when the animals are found in high densities (experience shortages of food) compared to the oval shell shape of animals growing under low densities. In addition, Breen and Adkins (1982) found that slow-growing *H. kamatsakana* populations had taller shells compared to fast-growing individuals, while Worthington *et al*. (1995) reported that slow-growing *H. rubra* populations in New South Wales had significantly wider shells than those of fast-growing abalone. Studies on shell morphology for the New Zealand black-footed abalone (*H. iris*) are lacking, although this species inhabits similar subtidal rocky shore habitats and feed on similar algal food sources as *H. rubra* (Australia) and *H. kamatsakana* (British Columbia). Thus, *H. iris* would be expected to have high morphological variability as its counterparts throughout the world. If different types and amounts of protein produce abalone with different shell morphologies, then cultured species may be manipulated to produce specific shell shapes for the jewellery market. In addition, shell morphology may be used as a proxy for animal health within farms and in the wild. Thus, the aims of this study are to identify the shell variations of *H. iris* juveniles grown with formulated diets containing different protein sources and amounts, and different temperature regimes.

6.3 Materials and Methods

6.3.1 Experimental design

Abalone (*H. iris*) were obtained from Seahorses Australasia Limited, Warrington Dunedin, New Zealand. Two sets of animals were used in two different experiments. For experiment 1, abalone of about 1.5 to 2 years old (20 to 22 mm in maximum shell length) were used to test the effect of different dietary protein sources on shell growth parameters. This experiment was conducted at Seahorses Australasia Limited. The experimental animals first were acclimatized for one month in a flow-through tank for one month, during which time they were fed a commercial diet (Adam and Amos abalone food, Australia). After acclimatization, the animals were carefully removed from the holding tank with a flat, blunt blade, and placed in the experimental tanks. Twenty randomly selected animals were placed in each of 27 experimental containers (1 L in volume with square PVC lids, semi-transparent in color). Three out of the 27 containers were randomly assigned one of nine dietary protein sources, which were fed to the animals daily for a five-month period. All the tanks were maintained with semi-recirculated (50% water exchange per day) filtered (10μm filter) seawater, and the temperature was kept constant at 18±0.3 °C throughout the experimental period.

For experiment 2, another set of animals (2 to 2.5 years old, 30 to 33 mm in maximum shell length) were used to test the effect of six different dietary protein levels and two temperature regimes on shell growth parameters. This experiment was conducted at the Portobello Marine Laboratory, University of Otago, Dunedin, New Zealand. For this experiment, five randomly selected animals were respectively placed in each of 36 experimental containers (1 L in volume with square PVC lids, semi-transparent in color) with flow-through seawater. The containers were arranged into two temperature regimes (18 containers per temperature regime). The high (13 to 21 °C) and the low (8 to 16 °C) temperature regimes were maintained throughout the four-month experimental period. For each temperature, three replicate tanks were assigned one of the six dietary protein levels.

For both experiments, the water quality always was kept in good condition (pH between 8.3-8.5, dissolved oxygen > 10 ppm). The daily feeding rates were 5% of the total body weight per tank, and the water flow rate was 500 ml/hour (total water exchange of 12 times per day). Faeces and uneaten food residues were swept away every other day.

6.3.2 Diet formulation

For experiment 1, nine diets were formulated to contain about the same nutritional profile, but using different sources of protein (white and red fish meals, blood meal, meat and bone meal, casein, soybean concentrate, wheat gluten, maize gluten and *Spirulina* powder) (Table 1). For experiment 2, six experimental diets were formulated to contain different levels of red fish meal protein (about 0, 10, 20, 30, 40, and 45%), while maintaining all other nutritional components similar (Table 2).

The formulation of both sets of diets involved the mixing of dried ingredients in a blender without the starch and cellulose. The starch was first mixed with boiling water in order to activate its binding propensities (gelatinize). Then, the activated starch and cellulose were added to the mixture of all other dry ingredients. Finally, lipid oil was added to make a dough, which was spread on a flat, square-sized board to a 3 mm thickness. Small pieces (1 cm^2) were cut with a knife just prior to drying in a Mcgreger hot-air oven at 45 °C for about 12 hours. The diets were then stored in a -20 °C freezer until they were used.

6.3.3 Shell analysis

Prior to the start of the experiments, measurements of maximum shell length and width were made for each individual animal (Fig. 1). At the end of each experiment (five and four months, respectively), all surviving animals were stripped of their shells with a flat, blunt blade. Broken shells were excluded from the analysis. A total of 16 to 33 shells per diet treatment was analyzed for the first experiment and 3-9 shells per treatment combination (diet and temperature) were analyzed for the second experiment. Individual shells were kept in 5% NaOH for 7 hrs to remove all organic material. Then, the shells were rinsed thoroughly with de-ionized water three times. After drying at 60 °C for 48 hrs, all the shells were measured to obtain their maximum shell lengths, widths and heights (Fig. 1) with a digimatic caliper with

accuracy of 0.01 mm (Mitutoyo Co., Japan). Shell thickness was measured at the edge of the first respiratory pore. Shell weight was measured with digital balance AB104-S with accuracy to 1 mg (Mettler Toledo Co., Japan).

6.3.4 Statistical analyses

Linear regressions were calculated between shell width, height, thickness and weight, against maximum shell length to test for linearity between the paired parameters. After corroborating linearity, shell width, height, and weight measurements were standardized by dividing each parameter by the shell length. Shell thickness was not divided by shell length because these two parameters did not follow a linear relationship. All data were checked for and met parametric assumptions. The shell growth parameters obtained from experiment 1 were analyzed with one-way ANOVAs (protein source as fixed factor), followed by Tukey *post-hoc* tests. The data obtained from experiment 2 were analyzed with two-way ANOVAs (diet and temperature as fixed factors) and Tukey *post-hoc* tests. All analyses were conducted using a MiniTab version 14 software package.

6.4 Results

Results from the initial shell measurements (prior to the start of the experiments) indicated that there were no significant differences in growth parameters (maximum shell length, and width) among diets for experiment 1, and among diets, temperatures and interaction for experiment 2 (Table 3). Thus, it was assumed that all growth differences observed at the end of the experiments were a product of treatment effects. For both experiments, linear relationships were found between shell width, height, and weight, and shell length, but not between shell thickness and shell length (Table 4).

6.4.1 Experiment 1

The shell morphology varied significantly among the diet treatments after the five-month experiment (Fig. 2-3). The mean (±SD) shell length, width, height, thickness, and weight

across all treatments were 27.22±2.11 mm, 18.60±1.58 mm, 6.29±0.45 mm, 0.66±0.07 mm, and 0.76±0.20 g, respectively, with ranges from 24.39 to 29.70 mm, 16.46 to 20.29 mm, 5.73 to 7.00 mm, 0.53 to 0.72 mm and 0.46 to 1.00 g, respectively. Significant linear regressions were found between shell length and all other shell parameters except for thickness (Table 4).

The highest means (±SD) width/length, height/length, and weight/length ratios were 0.696±0.002 (casein), 0.241±0.003 (casein), and 0.034± 0.004(white fish meal), respectively. The lowest means (±SD) width/length, height/length, and weight/length ratios were 0.673±0.003 (meat and bone), 0.217±0.004 (red fish meal), and 0.019±0.002 (blood meal), respectively. One-way ANOVAs for these ratios resulted in significant differences among the diets (Table 5). Since shell thickness did not follow a linear relationship with shell length, shell thickness alone was used in the analysis. After the five-month experimental period, abalone fed both fish meal, casein and meat and bone meal had significantly thicker shells, and abalone fed blood meal had significantly thinner shells than animals fed the other diets (Fig. 3, Table 5).

6.4.2 Experiment 2

Results from the second abalone culturing experiment with six diets with varying amounts of protein and two different water temperatures are shown in Figures 4-5. After the four-month experiments, the mean (±SD) shell length, width, height, thickness, and weight across all treatments were 35.73±1.64 mm, 24.53±1.38 mm, 8.00±0.47 mm, 0.87±0.05 mm, and 1.72±0.29 g, respectively for high temperature treatments were 35.98±2.00 mm, 24.65±1.65 mm, 8.15±0.54 mm, 0.90±0.04 mm, and 1.81±0.33 g, respectively for low temperature treatments were 35.48±1.33 mm, 24.41±1.19 mm, 7.85±0.36 mm, 0.83±0.03 mm, and 1.63±0.25 g. The ranges for shell length, width, height, thickness, and weight of animals cultured at high temperatures were from 33.30 to 37.74 mm, 22.32 to 26.04 mm, 7.36 to 8.81 mm, 0.86 to 0.97 mm and 1.38 to 2.17 g, respectively, and from 33.79 to 37.00 mm, 22.99 to 25.84 mm, 7.32 to 8.24 mm, 0.79 to 0.88 mm and 1.33 to 1.90 g, respectively, for those grown at low temperatures. Significant linear regressions were found between shell length and all other shell parameters except for height and thickness (Table 4).

The highest means (±SD) width/length, height, and weight/length ratios were 0.700±0.005 (40% protein at high temperature), 8.81±0.48 mm (30% protein at high temperature), and 0.575±0.022 (30% protein at high temperature), respectively, while the lowest means (±SD) values were 0.670±0.005 (0% protein at high temperature), 7.32±0.50 mm (0% protein at low temperature), and 0.039±0.002 (10% protein at low temperature), respectively. Two-way ANOVAs for these ratios resulted in significant differences among the diets for shell width/length and weigh/length, and between temperatures for weight/length ratios only (Table 6). Shell thickness again was analyzed alone, and resulted in significant differences between temperatures only (Fig. 5, Table 6).

6.5 Discussion

In this study, different diets (sources and amounts of protein) had significant effects on most shell morphology parameters, but temperature had little effect on shell morphology. Specifically, shell width varied greatly with the different protein sources and amounts in the feed, while shell height and weight were highly variable with protein source and shell weight varied among diets with different protein amounts. Conversely, shell thickness did not appear to vary much with different sources and amounts of protein. It is possible that such dietary differences may reflect the morphological variations observed among wild abalone populations, and that shell morphology may be regulated in aquaculture environments by adjusting the source and amounts of protein in the diets.

6.5.1 The relationship between food and shell morphology

Abalone have been shown to have strong morphological variability in different coastal habitats (Breen and Adkins, 1982; Shepherd and Hearn, 1983; Worthington et al., 1995; Wells and Mulvay, 1995). It is believed that differences in food supplies regulate abalone growth, especially of shells (Day and Fleming, 1992; Saunder et al., 2009). For example, nutritional experiments on *H. discus hannai* have shown that feeding animals with different seaweed species results in distinctive shell growth variations (Uki and Watanabe 1986). Such increase in growth with high food supplies may indicate that the animal has more energy to allocate for shell growth and/or that a more balanced nutritional supply is needed to produce an "optimal" shell shape. However, the relationship between diet and shell morphology still needs further investigation.

6.5.2 Effect of dietary protein source on shell morphology

In the present study, shell morphology was shown to be affected by the type of dietary protein in the five-month experiment. Among the nine different sources of dietary protein, the casein diet produced animals with the widest, highest and heaviest shells, while the white fish meal diet yielded a heavier shell and the red fish meal diet produced the flattest shell shape. Similar results were found by Uki and Watanabe (1986a), where *Haliotis discus hannai*

increased shell length with casein diets compared with fish protein. In the present study, all nine diets had similar nutritional values (e.g., similar protein, lipid and carbohydrate contents); it is suggested that the shell differences are a result of the animals incorporating different amino acids from the different food sources (i.e., different protein sources contain different amino acid profiles). For example, Mai et al. (1994) found that different amino acids produced different shell growth rates in *Haliotis discus hannai*. In the present study, animals fed blood meal produced significantly narrower, thinner, and lighter shells compared to those of abalone fed the other diets. These results indicate that blood meal is a poor diet for *H. iris*, and that these animals may have experienced negative growth during the five-month experimental period. While there is no prior information about the effect of blood meal on growth of abalone, blood meal has been shown to be a poor protein source for prawns, since it is difficult to digest (Brand and Colvin 1977).

A change in shape to a wider and taller shell may increase the volume to accommodate the soft tissues, including muscles used for attachment and movement, and may indicate good growing conditions. McCarthy et al. (2004) found that an increase in shell width of the periwinkle *Littorina saxatilis* resulted in a 12% increase in the total volume inside the shell. In addition, Bertness and Cunningham (1981) reported that snail with wider shells experience lower mortality rates than those with thinner shell. Thus, allocating more resources to the shell may indicate better growing conditions and may enhance survival rates.

6.5.3 Effect of dietary protein amount on shell morphology

Results from the second experiment indicate that high protein content (40%) in the diet tends to produce wider and heavier shells than foods with less protein. However, shell height and thickness do not appear to be affected by different dietary protein amounts. In this study, a wider and heavier shell, as a response to high protein diets, may be associated with the development of a stronger (bigger) muscle (Trueman and Brown, 1989), and may result in a stronger shell resistance against predators (Bertness and Cunningham 1981). Thus, the findings of this research support the idea that high protein diets enhance shell growth and improve the health and survival of abalone. These findings also are in agreement with Mai et al. (1995b) who found an increase in shell growth with increasing dietary protein level for *Haliotis tuberculata* and *Haliotis discus hannai*. In addition, Worthington et al. (1995) found

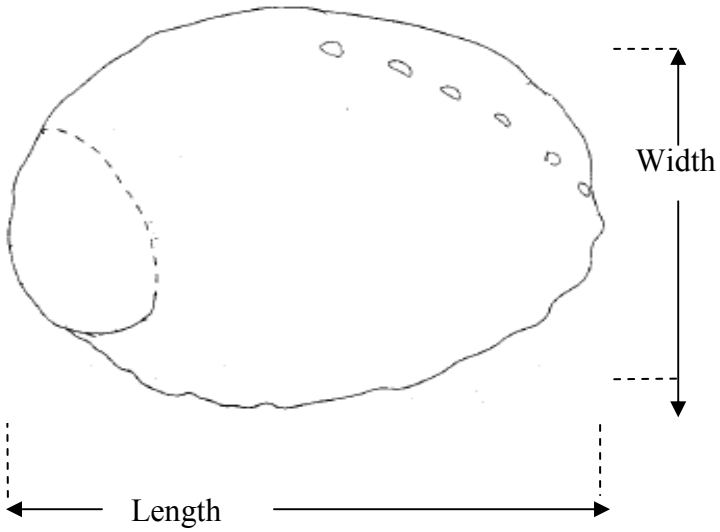
that wild populations of *H. rubra* in New South Wales, Australia, had wider shells in areas of low drift seaweed abundance compared to areas with high drift algal abundance. The reason for this discrepancy may be due to the fact that in areas of low macroalgal abundance, abalone tend to feed on microalgae which have higher protein contents (Tahil and Juinino-Menez, 1999). For example, drift seaweed normally contain 7-24% protein (Emmanuel and Corre, 1996; Barbarino and Lourenco, 2005), while many microalgal biofilms contain 41.80-50.85% protein (Nagarkar et al., 2004).

6.5.4 Effect of temperature on shell morphology

Temperature also has been shown to affect the shell growth of many marine invertebrates. For example, shell thickness of *Cypraea annulus* in Okinawa Island was found positively correlated with temperature (Irie, 2006). In the present study, *H. iris* juveniles exposed to high temperatures had thicker and heavier shells across different diets, but temperature did not affect shell width and height. It has been shown that in low temperatures, calcium carbonate solubility decreases, thereby increasing shell deposition and increasing shell thickness (Trussel and Smith, 2000). In addition, under low temperature, the precipitation of calcium carbonate increases on shell surfaces (Graus, 1974; Clarke, 1983). Thus, this "passive" shell deposition as a response to environmental temperature may explain the increase in shell thickness and weight, while "active" growth may be required for directional shell width and height increases. While snail shell morphological variations have been reported to occur as a response to different environmental temperatures, such studies have not been conducted for abalone until now. These finding may be useful in aquaculture situations where shell morphology needs to be manipulated (i.e., shell jewellery products), and/or to monitor wild abalone growth and health among different environmental conditions.

Figure 6. 1 Diagram indicating the measurement obtained from abalone shells (a) top view of shell (b) side view of shell.

(a)



(b)

Figure 6. 2 Mean (±SD) shell (a) width/length and (b) height/length of abalone after five-month cultivation with nine different dietary protein sources (WF = white fish meals, RF = red fish meals, BL = blood meal, MB = meat and bone meal, CA = casein, SO = soybean concentrate, GL = wheat gluten, MZ = maize gluten, and SP = *Spirulina* powder. Different letters on top of the error bars denote significant differences resulting fromTurkey *post-hoc* tests ($p < 0.05$).

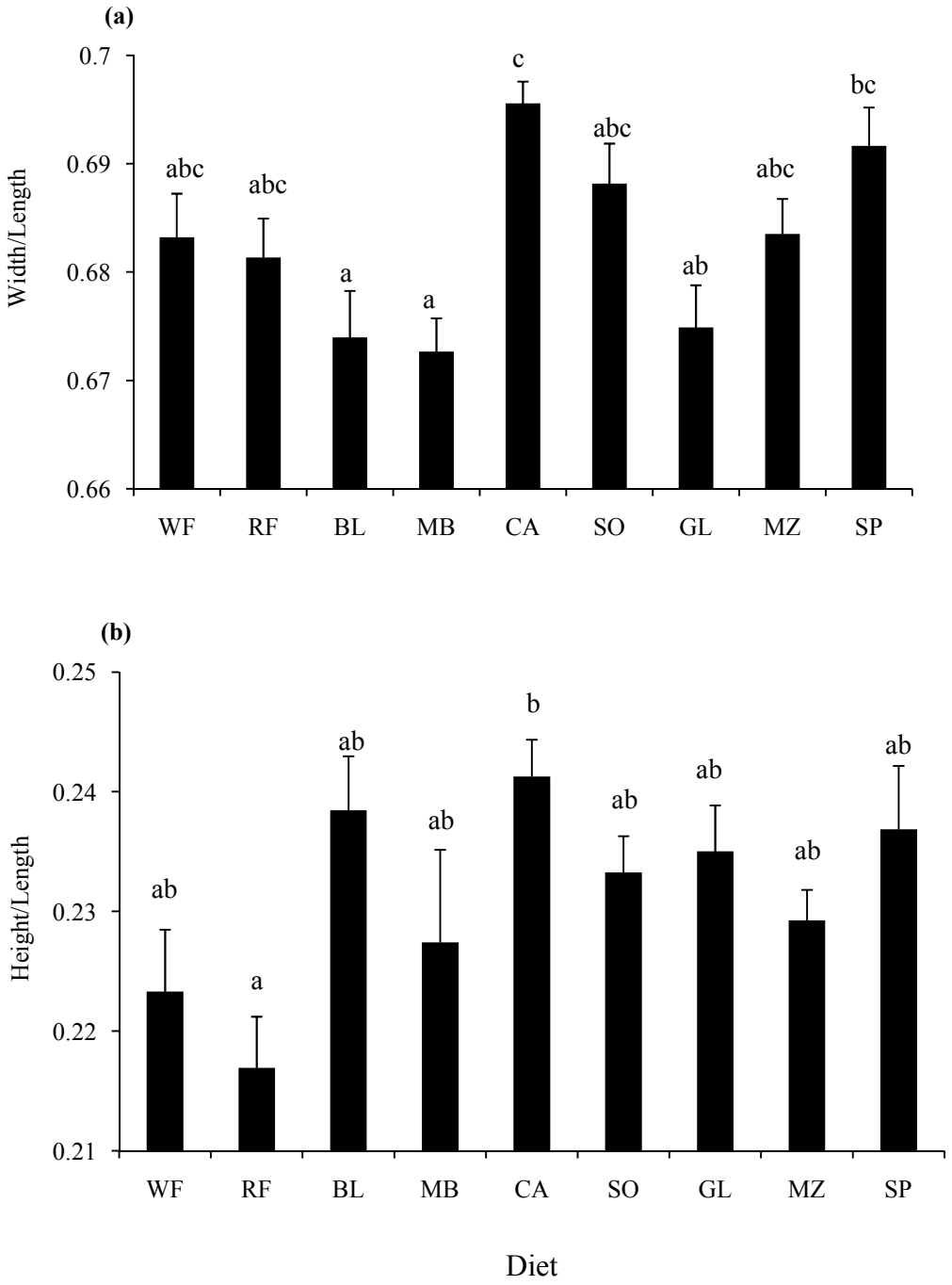


Figure 6. 3 Mean (±SD) shell (a) thickness and (b) weight/length of abalone after five-month cultivation with nine different dietary protein sources (WF = white fish meals, RF = red fish meals, BL = blood meal, MB = meat and bone meal, CA = casein, SO = soybean concentrate, GL = wheat gluten, MZ = maize gluten, and SP = *Spirulina* powder. Different letters on top of the error bars denote significant differences resulting fromTurkey *post-hoc* tests ($p < 0.05$).

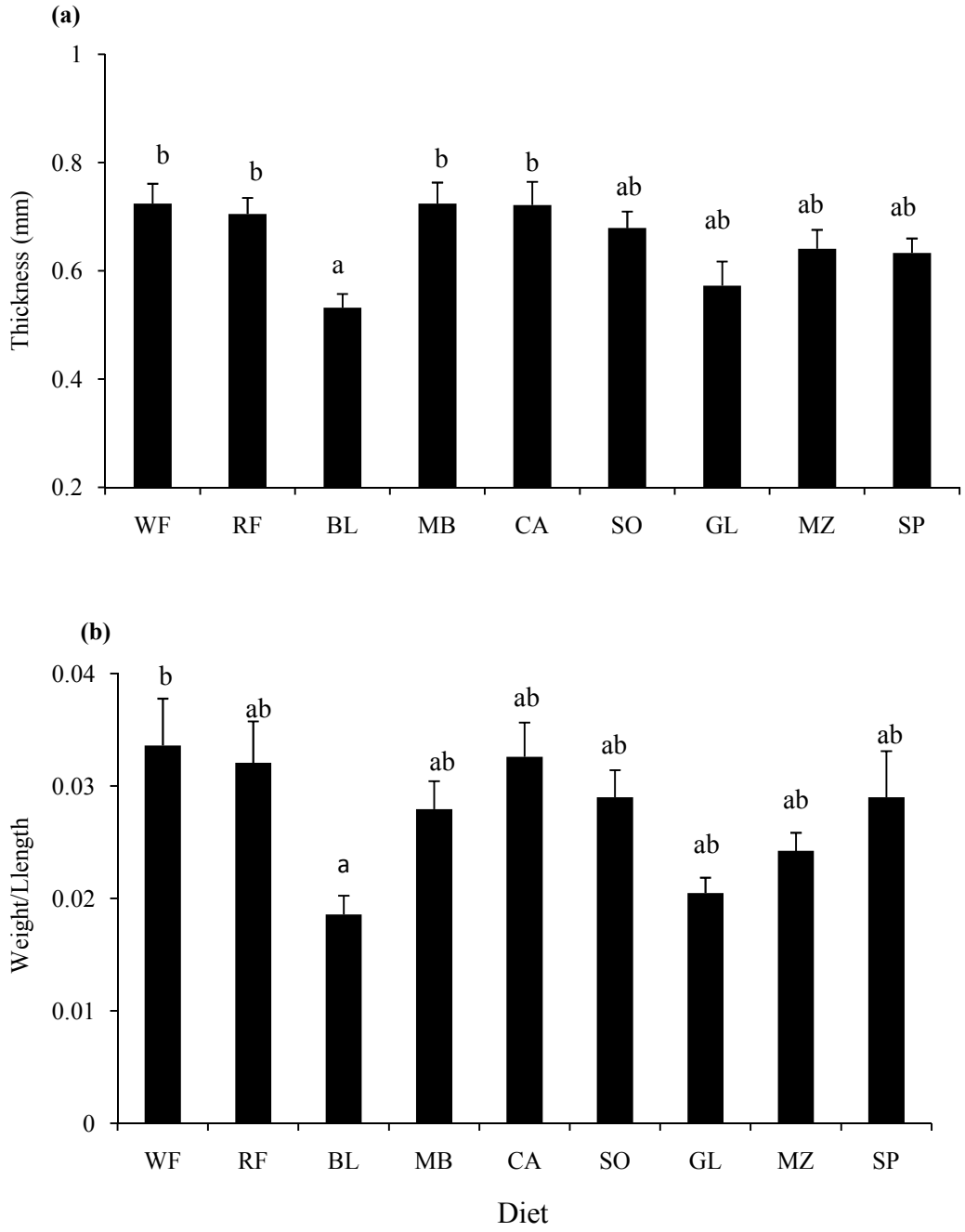


Figure 6. 4 Mean (±SD) shell (a) width/length and (b) height of abalone after four-month cultivation with six different dietary protein amounts (0, 10, 20, 30, 40, and 45% protein) diets and two temperature regimes. Data are presented as means±SD of all animals in treatments. Different letters on top of the error bars denote significant differences resulting fromTurkey *post-hoc* tests ($p < 0.05$).

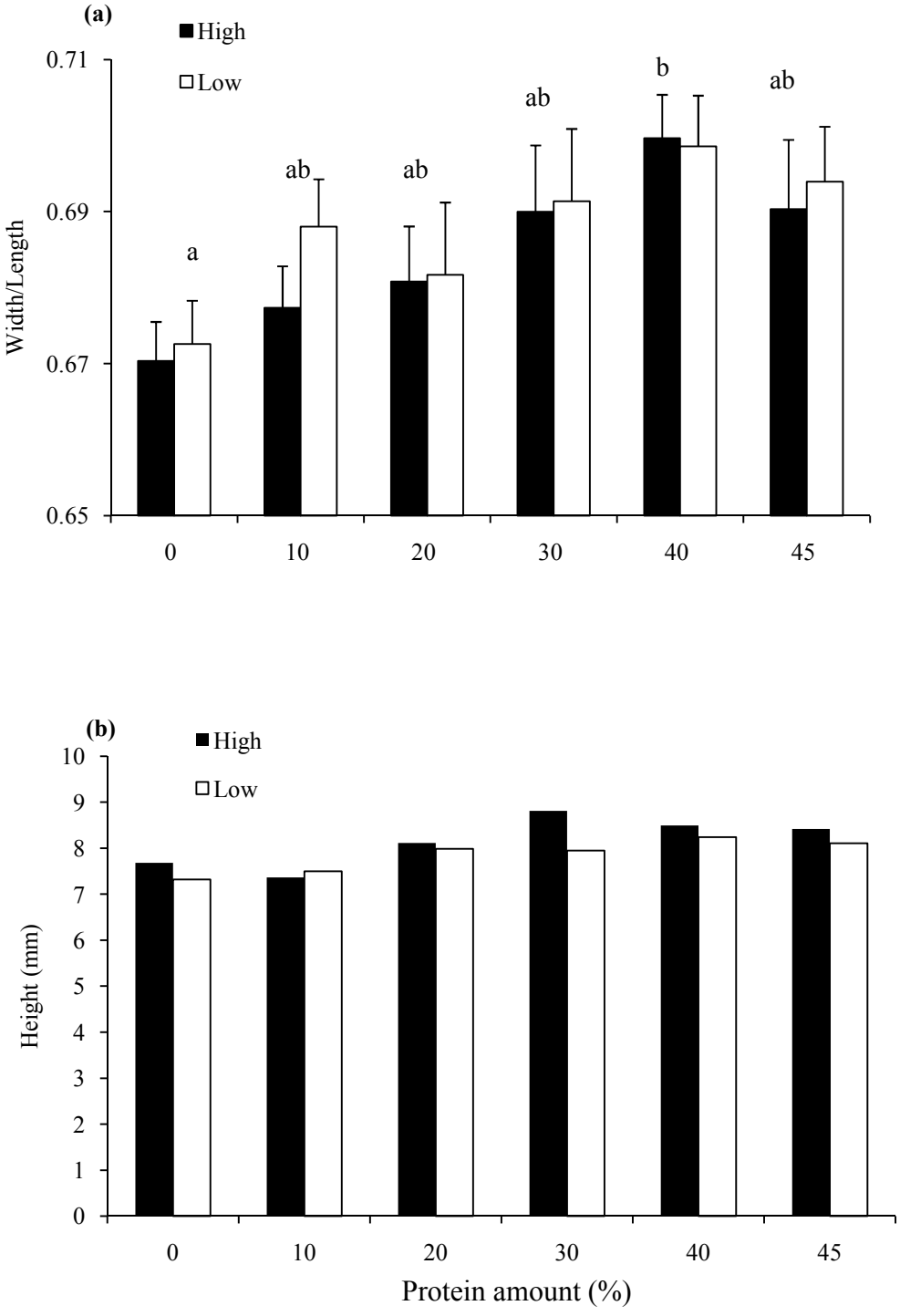


Figure 6. 5 Mean (±SD) shell (a) thickness and (b) weight/length of abalone after four-month cultivation with six different dietary protein amounts (0, 10, 20, 30, 40, and 45% protein) diets and two temperature regimes. Data are presented as means±SD of all animals in treatments. Different letters on top of the error bars denote significant differences resulting fromTurkey *post-hoc* tests ($p < 0.05$).

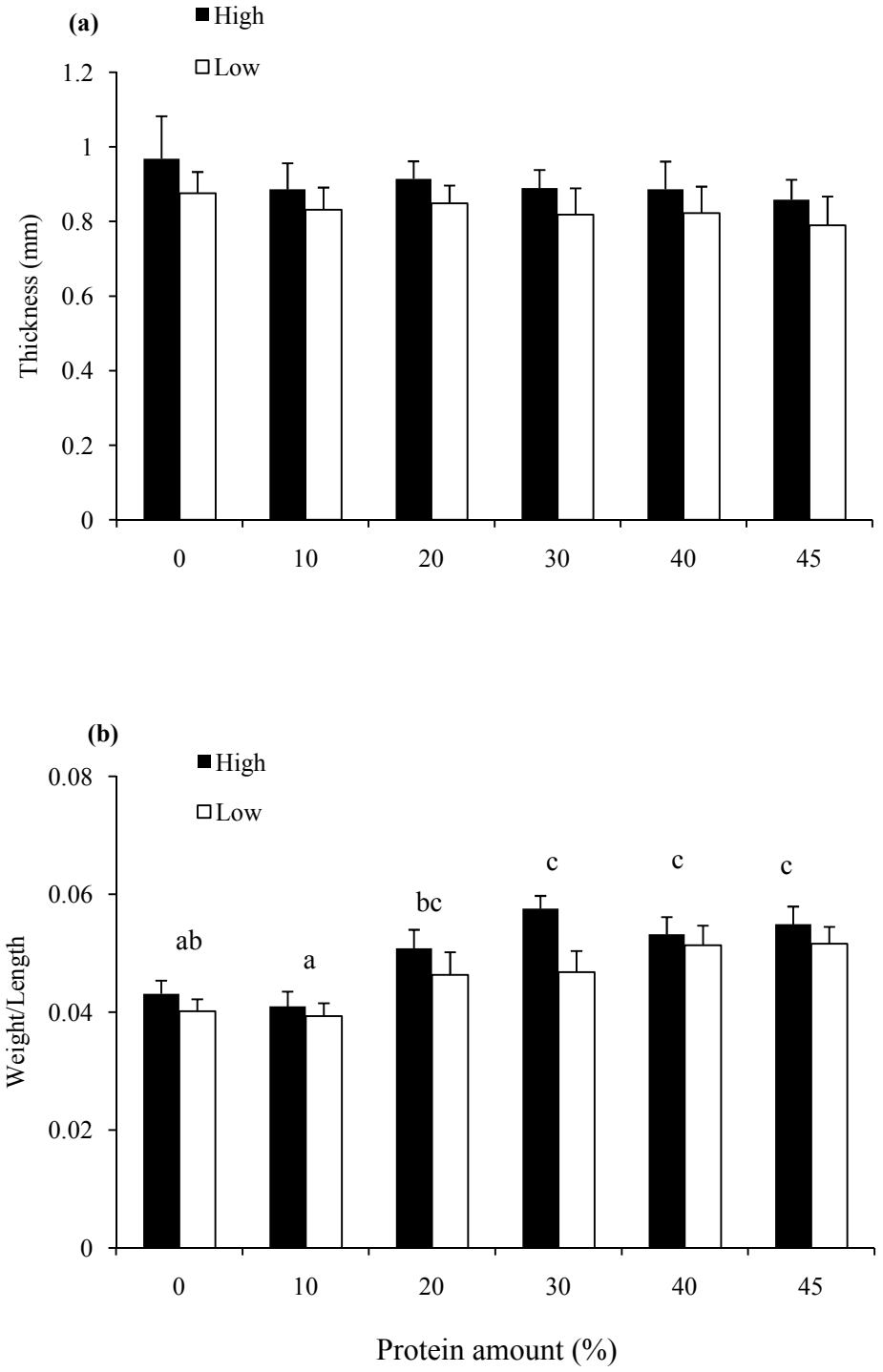


Table 6. 1 Recipes for nine experimental diets with different protein sources. The nine protein sources were white fish meals (WF), red fish meals (RF), blood meal (BL), meat and bone meal (MB), casein (CA), soybean concentrate (SO), wheat gluten (GL), maize gluten (MZ) and *Spirulina* powder (SP).

Diet	White fish meal[a] (WF)	Red fish meal[a] (RF)	Blood meal[a] (BL)	Meat and bone meal[a] (MB)	Casein[a] (CA)	Soybean concentrate[a] (SO)	Wheat gluten[a] (GL)	Maize gluten[a] (MZ)	*Spirulina*[a] (SP)
Protein (g/100g diet)	42.40	40.50	29.70	52.00	31.90	54.90	35.80	42.50	49.60
Cellose[b] (g/100g diet)	14.10	16.50	22.40	-	20.2	-	16.7	11.9	7.40
Starch[c] (g/100g diet)	40.0	40.00	40.00	40.00	40.0	40.0	40.0	40.0	40.00
Lipid[d] (g/100g diet)	0.50	-	4.90	-	4.90	4.10	4.50	2.60	-
Vitamine[e] (g/100g diet)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Minerial Mix[f] (g/100g diet)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Average pellet weight (g)	0.18	0.25	0.23	0.34	0.19	0.25	0.39	0.26	0.26

a. white and red fish meal supplied by Sealord, Nelson (NZ) LTD. Casein supplied by Anchar. Meat and bone meal, and blood meal supplied by Affco (NZ) Limited. Wheat gluten, maize gluten and soybean concentrates from J. C. Sherratt & Co. Ltd. *Spirulina* from Superfood Spirulina Ltd.

b. from commercial product "Just-Fiber ™".

c. modified starch (Firm-MIX ™) used for binding pellets from New Zealand Starch LTD.

d. cold-filter cod liver oil (Healther™, NZ).

e. Vitamin mix (Shin- Chan™, Taiwan) per g content: Riboflavin 10mg, PABA 40mg, Pyridoxine HCl 4mg, Niacin 80mg, Ca pantothenate 20mg, Inositol 400mg, Ascorbic acid 400mg, Biotin 1.2mg, Vitamin E 45mg, Menadione 8mg, Vitamin B_{12} 18mg, Vitamin A 10000 I.U., Vitamin D 200 I.U., Ethoxyquin 40mg, Folic acid 3mg, Thiamin HCl 12mg.

f. Mineral mixture g/kg mixture: NaCl (BDH) 10g, $MgSO_4 \cdot 7H_2O$ (BDH) 10g, $NaH_2PO_4 \cdot 2H_2O$ (BDH) 150g, KH_2PO_4 (BDH) 250g, K_2HPO_4 (BDH) 320g, $Ca(H_2P0_4)_2 \cdot H_2O$ (Merck) 200g, Fe-lactate (SIGMA) 25g, Ca-lactate (SIGMA) 35g, $ZnS0_4 \cdot 7H_2O$ (BDH) 3.53g, $MnS0_4 \cdot 4\ H_2O$ (BDH) 1.62g, $CuSO_4 \cdot 5H_2O$(BDH) 310mg, $CoC1_2 \cdot 6H_2O$ (Merck) 10mg, KIO_3 (Merck) 30mg

Table 6. 2 Recipes for diets with different amounts of protein. Data are presented as means of three replicate samples. Six diets were formulated with different protein amounts (1= 0%, 2= 10%, 3= 20%, 4= 30%, 5= 40%, and 6= 45%).

Diet number	*1*	*2*	*3*	*4*	*5*	6
Protein level	0%	10%	20%	30%	40%	45%
Red fish meal[a]	-	9.6	19.6	29.6	39.6	44.6
Cellose[b]	49.8	41.5	32.8	24.1	15.4	11.7
Starch[c]	40.0	40.0	40.0	40.0	40.0	40.0
Lipid[d]	7.2	5.9	4.6	3.3	2.0	0.7
Vitamine[e]	1.5	1.5	1.5	1.5	1.5	1.5
Minerial Mix[f]	1.5	1.5	1.5	1.5	1.5	1.5
Average pellet weight (g)	0.19	0.22	0.23	0.25	0.28	0.31

a. red fish meal supplied by Sealord, Nelson (NZ) Ltd..

b. from commercial product "Just-Fiber ™"

c. modified starch (Firm-MIX ™) used for binding pellets from New Zealand Starch Ltd.

d. cold-filter cod liver oil (Healther™, NZ).

e. Vitamin mix (Shin- Chan™, Taiwan) per g contain: Riboflavin 10mg, PABA 40mg, Pyridoxine HCl 4mg, Niacin 80mg, Ca pantothenate 20mg, Inositol 400mg, Ascorbic acid 400mg, Biotin 1.2mg, Vitamin E 45mg, Menadione 8mg, Vitamin B_{12} 18mg, Vitamin A 10000 I.U., Vitamin D 200 I.U., Ethoxyquin 40mg, Folic acid 3mg, Thiamin HCl 12mg.

f. Mineral mixture g/kg mixture: NaCl (BDH) 10g, $MgSO_4 \cdot 7H_2O$ (BDH) 10g, $NaH_2PO_4 \cdot 2H_2O$ (BDH) 150g, KH_2PO_4 (BDH) 250g, K_2HPO_4 (BDH) 320g, $Ca(H_2P0_4)_2 \cdot H_2O$ (Merck) 200g, Fe-lactate (SIGMA) 25g, Ca-lactate (SIGMA) 35g, $ZnS0_4 \cdot 7H_2O$ (BDH) 3.53g, $MnS0_4 \cdot 4\ H_2O$ (BDH) 1.62g, $CuSO_4 \cdot 5H_2O$(BDH) 310mg, $CoC1_2 \cdot 6H_2O$ (Merck) 10mg, KIO_3 (Merck) 30mg.

Table 6. 3 (a) One-way ANOVA analyses of nine diets in experiment 1 for initial shell length and width measurements, and (b) two-way ANOVA analyses of six diets and temperatures in experiment 2 for initial shell length and width measurements.

Experiment 1

		Length				*Width*		
Source	df	MS	F	p	df	MS	F	p
Diet	8	0.43	1.11	0.359	8	0.11	1.11	0.366
Error	18	0.38			18	0.10		
Total	26				26			

Experiment 2

		Length				*Width*		
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.27	0.18	0.673	1	0.13	0.18	0.674
Diet	5	0.72	0.47	0.795	5	0.34	0.47	0.796
Interaction	5	0.50	0.32	0.897	5	0.23	0.32	0.900
Error	24	1.52			24	0.72		
Total	35				35			

Table 6. 4 Linear regression results of shell length to four shell parameters (width, height, thickness, and weight) for experiments 1 and 2. Bold values denote significant differences ($p < 0.05$).

	Experiment 1			*Experiment 2*		
	Standard coefficient	T value	p value	Standard coefficient	T value	p value
Width	0.74	19.77	**0.001**	0.71	8.86	**0.001**
Height	-0.04	-2.13	**0.030**	-0.02	-0.39	0.700
Weight	0.29	6.53	**0.001**	0.28	2.99	**0.001**
Thickness	-0.20	-1.15	0.250	0.03	0.78	0.440

Table 6. 5 One-way ANOVA analyses among diets for shell width/length, height/length, weight/length and thickness. Bold values denote significant differences.

	Width/Length				*Height/Length*			
Source	df	MS	F	p	df	MS	F	p
Diet	8	0.00	5.10	**0.002**	8	0.00	2.82	**0.032**
Error	18	0.00			18	0.00		
Total	26				26			
	Thickness				*Weight/Length*			
Source	df	MS	F	p	df	MS	F	p
Diet	8	0.02	4.00	**0.007**	8	0.02	3.36	**0.016**
Error	18	0.00			18	0.00		
Total	26				26			

Table 6. 6 Two-way ANOVA analyses among diets and temperatures for shell width/length, height, thickness, and weight/length. Bold values denote significant differences.

	Width/Length				*Height*			
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.00	0.48	0.494	1	0.80	1.32	0.262
Diet	5	0.00	3.52	**0.016**	5	1.11	1.83	0.145
Interaction	5	0.00	0.15	0.977	5	0.16	0.27	0.926
Error	24	0.00			24	0.61		
Total	35				35			

	Thickness				*Weight/Length*			
Source	df	MS	F	p	df	MS	F	p
Temperature	1	0.05	5.01	**0.035**	1	0.00	6.19	**0.020**
Diet	5	0.01	0.75	0.591	5	0.00	7.84	**0.001**
Interaction	5	0.00	0.04	1.000	5	0.00	0.67	0.647
Error	24	0.01			24	0.00		
Total	35				35			

Chapter 7 General Discussion

The cultivation of the New Zealand black-footed abalone (*H. iris*) is on the way toward artificial formulated diets. However, published information is lacking on the nutritional requirements for this species. This lack of knowledge seems likely to have been delaying the development and progress of the country's black-footed abalone aquaculture industry for years. The aim of the present study was to investigate the role of dietary proteins and amino acids on *H. iris*, in both larval and juvenile stages, which in turn could help abalone farmers to culture or manufacture better diets for this species.

Twenty young and mature microalgal biofilms isolated locally were tested for their effectiveness on *H. iris* larvae settlement. Amino acid profile and percent coverage of microalgal strains were also monitored. Little difference was observed on settlement parameters between the different strains of microalgal biofilms. However, a significant correlation of larval attachment and metamorphosis was found on mature biofilms between total amino acids (TAA) levels and percentage coverage of microalgal strains. The amino acid profile seemed to have no effect on larval settlement. These results indicate the importance of dietary protein in *H. iris* larval culture especially with regard to protein content. However, the role of more than one species in the microalgal biofilm is more likely to meet the balance of amino acid profiles. However more studies are needed in this area of research.

Out of the 20 strains used in experiment in Chapter 2, 9 of those strains were randomly mixed to produce dual-strain microalgal biofilms, and their effect on settlement of *H. iris* larvae was monitored. The mixed dual-strain microalgal biofilm was more likely to meet the balance of amino acid profiles which could boost settlement, as was suggested by Gordon et al. (2006). However, there was no clear relationship found between the amino acid profile and the larval settlement parameters. Adding some cynobacterium strains to the dual-strain microalgal biofilm improved the larvae settlement significantly as was shown from the result of high TAA level. In both the above larvae settlement experiments, correlation analysis indicated that dietary protein content (i.e. TAA) may be more important than composition of the dietary protein in *H. iris* larvae culture.

The next step was to explore suitable protein sources for *H iris* in formulated diets with a view to find the best sole protein source for its aquaculture. Due to commercial aspects, the proteins that were to be used in the artificial diets had to be economically viable, easily accessible, and provide a fast growing effect on the target species. Therefore, for this work, nine easily accessible proteins in New Zealand were chosen as sole protein sources, which included some plant proteins with unbalanced amino acid profiles. These plant proteins generally have a better binding ability than animal protein, which led to high water stability of the artificial diets. The plant proteins also were generally more economical than the animal protein. Further investigations also conducted to see whether diets could lead to amino acid deficiency by analysing shell protein and soft body tissue amino acid compositions. As was observed previously with larval settlement, the amino acid profile did not affect the growth of juvenile *H. iris*.

Most of the introduced diets (white fish meal, red fish meal, casein, soybean protein and *Spirnalina* protein), containing different amino acid profiles, had similar growth effects on juvenile *H. iris*. Of the five protein diets, soybean protein, which contained extremely low methionine in the protein, still produced similar growth and survival rate as fish protein. Even with low methionine content in the soybean protein, similar methionine content as animals fed fish protein diet also was found in the tissue of abalone that fed soybean diet. From the results of the five diets (mentioned above), on growth and health parameters, red fish meal proved to be the most obvious choice as the sole protein source in order to test protein requirement in the proceeding work.

Next, the relationship between different temperature regimes and abalone protein requirements was tested. Temperature was simulated as average surface sea temperature of North Island and South Island in New Zealand in order to find an acceptable protein contents that would be suitable all over New Zealand. Different variables (e.g. soft tissue, shell and histological features of abalone) were compared in different dietary treatments. The amino acid composition of shell protein and soft body tissue also was analysed to compare the effect of temperature on protein levels. Second-order polynomial curves were used to estimate the protein requirement under different temperatures. The results suggested that *H. iris* require more protein at low temperature than high temperature environments. The decreasing growth in colder environments can be overcome by increasing dietary protein rather than heating culturing water.

Results from the shell morphological measurements, showed that amino acid profiles and environment temperature not only affect the amino acid profile in the shell, but also affect the morphology of the shell.

In conclusion, dietary protein is an important factor for abalone aquaculture. The content of dietary protein strongly affects settlement of larvae and growth of juveniles in *H. iris*. For large scale *H. iris* aquaculture, 40 to 45% of dietary protein content, as was provided by red fish meal in the artificial diets, is recommended from the results of this study. Raising dietary protein contents can effectively recover the decrease of growth that results from low water temperature.

7.1 The most preferred alternative protein in *H. iris* diet

At present, New Zealand is relying on importing dietary products for abalone from Australia and South Africa. However these diets are designed for other abalone species which may not satisfy the nutritional requirements for *H. iris*. According to Fleming et al. (1996), the only commercial products present in New Zealand uses casein as the main protein sources. However, 53% of the crude protein content may not be cost-effective product, according to the results of this present study. According to the results from Chapter 4, the best dietary protein that generated high growth, more muscle tissue, good health, and high survival for animals was red fish meal. Casein proved to be nearly twice as expensive as red fish meal, even though New Zealand is the main casein producing country in the world. In the present study, red fish protein in the diet produced the highest animal mean protein gain, which indicates that this diet is readily digested by *H. iris*. The high mean protein gain also resulted in the highest soft body/shell ratio, indicating that the fish meal protein diets for animals produced more muscle (higher soft body/shell rations) than animals fed on other diets. These results suggest that cultivation of *H. iris* as a commercial canned product (meat without shell) may benefit from feeding the stock with a high amount of fish meal protein in their diets. Previous studies on other abalone species also confirm that fish meals generally yield good animal weight gains (Ogino and Kato, 1964; Britz, 1996b).

However, one potential alternative protein source highlighted in Chapter 4 is soybean protein, which also yielded good growth rates. Replacing fish meal protein with soybean protein did not significantly decrease growth rates. Therefore, since soybean protein is much

more economical than fish meal protein, reducing overhead costs in diets by replacing economical protein source in diets could greatly benefit abalone farm owners. In addition, the soybean protein diet produced animals with the heaviest gonads and highest gonad/soft body index (GSI) among animals fed with different diets. The reason for the greater gonad development with this protein source may be related to the relatively high amount of poly-unsaturated fatty acids (about 47% of total FAA) provided by the soybean diet. Poly-unsaturated fatty acids also were found to improve the GSI and reproductive activity in the freshwater swordtail fish, *Xiphophorus helleri* (Lin and Janz, 2006). It is assumed here that cultivation of *H. iris* for broodstock purposes may benefit from feeding the stock with a replacement of a high proportion of fish meal with soybean protein in their diets.

However, soybean contains less protein and more fibres, thus, making it less palatable and more difficult to reach the 40% crude protein criteria that was suggested in Chapter 5. Soybean protein, therefore, needs to be coupled with a small proportion of binding proteins such as maize protein or wheat gluten. A combination of these three ingredients (soybean, maize and wheat gluten) may provide a useful replacement for red fish meal in diets. This may still require further experimental work.

7.2 Dietary protein requirements

The dietary protein requirement experiment in Chapter 5 detected the most economical amount of protein for optimal growth in *H. iris* juveniles. The result from Chapter 5 showed that a 40% protein diet provides the best performance for the growth and health of the animals. Second-order polynomial regression curves for body weight and protein gain also showed best performance within a range of 38-54% dietary protein, depending on the temperature regime and the biological parameters used. This type of regression curve is commonly used to identify nutrient and protein requirements for aquaculture species, such as the abalone *H. discus hannai* and *H. tuberculata* (Mai et al., 1995b) and *H. laevigata* (Coote et al., 2000). In the present study, the regression curves suggest that animals exposed to higher temperatures would be expected to have lower protein requirements (about 38%), and those exposed to lower temperatures would be expected to have higher protein requirements (about 42-54%, depending on the moisture of animals). Therefore, at least 40% of dietary protein may be needed for cultured *H. iris* in order to satisfy the different water temperatures in New Zealand.

From this study, it can be concluded that the protein requirement of abalone may change under different environmental temperatures. *H. iris* tends to require more protein while living in colder environment. However, Mai et al. (1995b) and Coote et al. (2000) reported the protein requirement values for other abalone species only were tested at one temperature regime. For example, based on second-order polynomial regression curves, *H. discus hannai* and *H. tuberculata* require 35.6 and 32.3% protein, respectively, at 13-15 °C (Mai et al., 1995b), and *H. laevigata* requires 27% protein at 20 °C (Coote et al., 2000).

With at least 40% of protein (red fish meal), less than 5% of lipid (cod liver oil) and 40% of starch (which was used in the present study), this combination could ensure reasonable water stability of artificial diets and good growth rates of animals. This dietary combination can suit almost all the land-based farms anywhere in New Zealand without changing water temperature, including the South Island (where water temperature had to be raised by heating in winter using other diets). In the case of the South Island, the maintenance of rapid growth in low water temperature environments may be achieved by using a flow-through system instead of a recirculation seawater system.

According to Davis and Carrington (2005), an abalone farm on South Island in New Zealand has to spend $6000 in heating water during coldest four months in winter in order to produce 100kg abalone products from 40-50mm *H. iris* juvenile. Even if it adopts a new model of heating system, it still needs $538NZD of heating cost per year to produce 100Kg abalone product with a $7000NZD equipment investment. However, the better alternative, this study has suggested by raising up dietary crude protein level from 30% to 45% with red fish meal [$3.45/Kg] only in winter while the sea water temperature below 10 °C, would achieve nearly double growth rate compared with water- heating farm. This is due to high water exchange that does not require any heating sea water. And non-water-heating farm suggested by this study only costs extra $91NZD.6 per year to produce 100kg abalone products from 30-40mm animals. Better still, no heating equipment requirement is necessary for this type of farm. This non-water-heating farm promises to save 83% energy cost in comparison with those new-model heating system, and more than 98% energy cost in comparison with the traditional heating system.

Dietary protein levels (by means of total amino acids contents [TAA] of biofilm in Chapter 2 and 3) strongly affected the attachment and metamorphosis of *H. iris* larvae. In both mature single strained and dual strained biofilms, which contain higher TAA, showed better effect on all settlement parameters. The reason behind this relationship remains unclear. One possible reason is that the higher amino acid content partially contributed to a higher amount of ECP being produced by microalgae or bacteria in the biofilms. These ECPs have been shown to accumulate in biofilms over time (Batté et al., 2003). The amount of ECPs was found significantly positively correlated with the larval settlement of *H. diversicolor* (Chen, 2007). However, the abundance of bacteria and amount of ECPs were not monitored in this study. Their actual relationship may need further investigation.

7.3 Temperature

From this study it was clear that abalone changed their protein requirements under different water temperatures. It was also found that the growth decrease observed under low temperatures can be recovered by simply increasing dietary protein levels. This is a very economy method for farmers to grow abalone especially for those farms that are located in the South Island. By providing 45% of the dietary protein to the animal cultured at 10°C, similar growth performances can be achieved to animals cultured at 15°C.

Abalone grown in the low temperature regimes also had a significantly greater number of goblet cells and thicker epithelial layers than those grown in high temperature regimes. Previous studies have shown that gastropods are less mobile during winter (colder temperatures) and produce higher protein mucous to increase attachment strength (Donovan, 1998; Smith and Morin 2002). This may partially explain why they require higher protein in their diet under lower temperature. However, due to the digestion efficiency, metabolic rate and mucus production were not monitored in the present study, the actual metabolic function that *H.iris* juvenile under low temperature was still unknown. Further study is required in order to clarify this relationship.

Another alternative option for hatchery farmers is to increase the temperature during the whole process of abalone settlement. Natural spawning of *H. iris* usually occurs in autumn and early winter, hence the reason why most farmers manipulate larval settlement in winter or spring during cooler water temperature. However, increasing culturing temperatures of settlement tanks is rarely performed in the industry. In conclusion, larval attachment and metamorphosis correlated strongly with micro-algal protein contents in microalgae and their percentage cover on the surface of water. If protein requirements for abalone post-larvae change in parallel with their juveniles in response to temperature, then increasing water temperature during the settlement period might improve larval settlement. Such increases in water temperature generally decrease the protein requirements, boost feed ingestion rates of larvae, and enhance micro-algal growth rates. Furthermore, higher temperatures improve the development of bacteria and biofilms that have proven to be a key factor for successful larvae settlement.

7.4 Amino acid profiles

The result of this study found that amino acid profiles of dietary protein did not exercise much effect on settlement of *H. iris* larvae. Results from Chapter 2 and 3, show amino acid profile parameter such as EAA, NEAA, EAA/NEAA did not correlate with settlement parameters of *H. iris* larvae. In the mixture experiment, while randomly mixing nine strains of microalgae, no boost effect was observed. In combination of the results of these two Chapters, *H. iris* settlement may not be related to the profile of amino acids.

Similar observation was found in Chapter 4. Most of the introduced diets (white fish meal, red fish meal, casein, soybean protein and *Spirnalina* protein) containing different amino acid profiles produced similar growth rates on juvenile *H. iris*. However, soybean protein which contains extremely low methionine still produced similar growth and survival as fish protein. All those results seem to indicate that dietary amino acid is used as the main energy source for boosting the growth of *H. iris* and that the metabolic function of different amino acids may be powerful enough to support a low requirement of some specific amino acid in diets. Similar observations also can be found in other abalone species. *H. kamfschtkana* when fed with glycine dominant artificial feeds has similar growth rate as when fed with other protein sources (Taylor, 1997). However, this requirement of amino acid varies among abalone species. For example, *H. asinina*, replacement of fish meal with 35% defatted soybean in diets and 20% *Spirulina* protein causes a reduction in growth and a methionine deficiency in soft body tissues (Bautista-Teruel et al., 2003). According to the results obtained in this thesis, all plant protein sources which contain low dietary methionine content still maintain similar high methionine levels in the soft tissue of juvenile *H. iris*. Even the diet with lowest methionine content (soybean protein) was still able to maintain similar growth as that observed with fish meal protein diets. This may be due to a lower methionine requirement of *H. iris*, which results in a much wider range of food sources that can sustain good growth in this species.

In the present study, different dietary amino acid profiles could produce similar growth and survival of *H. iris* juveniles. However, dietary amino acid profiles are strongly affected by the amino acid profile in the soft bodies and shells. Furthermore, when the amino acid profile was fixed, such as shown in Chapter 5 experiment, few differences were found in the amino acid profiles of soft bodies or shell between different dietary protein levels. This may

indicate that the amino acid profile in soft body tissue of *H. iris* is only affected by the dietary amino acid that is provided in the food. These results may provide useful implications for the aquaculture industry. For example, it may be possible to change the flavour of *H. iris* tissue by controlling the dietary amino acids profile (i.e. by adding extra sweet-tasting amino acids such as glutamic acid in artificial feeds).

7.5 Shell protein and morphology of the shell

According to the results of Chapter 4, the shell amino acid profile was strongly affected by dietary amino acid profile. Based on the acid/basic amino acid ratios, animals grown with casein protein had shells with the highest amount of calcite *versus* aragonite. Interestingly, in Chapter 6, *H. iris* juvenile shell morphology was greatly affected by protein sources, more precisely by amino acid composition. Casein diets generated the highest acid/basic ratio in shells as well as the widest and highest shell shape. In contrast, the lowest acid/basic ratios in shells were found after feeding animals with white fish diets, which also produced a longer and flatter shell shape. Whether there is a linkage between the acid/basic ratios (i.e. calcite-to-aragonite ratios) and shell shape is still unclear. However, monitoring the shell shape changes may possibly become a non-invasive technique for abalone pearl farmers. This non-invasive technique may also be beneficial to the abalone pearl farmer by controling the appropriate shape by providing different amino acid in diets to facilitate the insertion of pearl into the abalone.

7.6 Future research

The findings of this study raise many questions, which still need to be answered. Further research may be directed in the following areas:

Chapter 2

- Does temperature affect abalone larval settlement?
- A quantitative study on the biofilms' physical features such as their thickness and amount should be applied on good microalgal strains in order to identify the proper age and quality that are best for larval settlement.

- What role do bacteria play in biofilms for larval nutritional requirements?
- What role does ECPs in biofilms play in larval nutritional requirements?
- What kinds of ECPs are produced by different bacterial biofilms, and how do they affect *H. iris* settlement?

Chapter 3

- Quantitative study on the number change of bacteria and amount of ECPs should be applied while mixing two strains of microalgae.
- How do the nutrient values of other nutrients (such as carbohydrates, fatty acids) of biofilms change while mixing dual strains of microalgae and how do they affect *H.iris* larval settlement?

Chapter 4

- What is the mechanism for the relationship between diets containing *Spirulina* protein and high shell to soft-body ratio?
- What is the mechanism for the relationship between diets containing soybean protein and high GSI?
- What is the mechanism for the relationship between the acid/basic amino acid ratio in shell and dietary protein?
- Did the acid/basic amino acid ratio in shell change in response to changes in amount of specific shell protein, such as pearlin?
- What is the function of GABA in abalone shells?

Chapter 5

- Why do protein requirements change in different temperatures?
- When a high protein diet is provided, how are metabolism rates and oxygen consumption affected under low temperature regimes?
- Does digestive enzyme efficiency change under different temperatures?

Chapter 6

- What is the relationship between shell protein compositions, shape of abalone shells, and its crystallization features?
- Could the shape of shell be controlled by altering dietary protein compositions?
- What kind of relationship exists between different amounts of shell protein and different amino acids that are provided in diets?

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Appendex I Histological procedure and solution

Table I. 1. Staining procedures for Haematoxylin and Eosin (H&E) and Periodic Acid- Schiff (PAS)

Harris method for Haematoxylin and Eosin (H&E)		Periodic Acid- Schiff (PAS)	
Xylol	5 min (dewaxing)	Xylol	5 min (dewaxing)
Xylol	5 min (dewaxing)	Xylol	5 min (dewaxing)
100% (absolute) alcohol	1 min (hydration)	100% (absolute) alcohol	1 min (hydration)
95% alcohol	1 min	95% alcohol	1 min
95% alcohol	1 min	95% alcohol	1 min
70% alcohol	1 min	70% alcohol	1 min
Wash in running tap water		Wash in running tap water	
Haematoxylin (Harris's)	8 min	0.5% periodic acid	5 min
Tap water	20 sec	Wash in running tap water	
Blue' in Scott's solution	1 min	Schiff reagent	10 min
Wash in tap water	2 min	Wash in tap water	
Eosin	3-5 min	Mayer's haematoxylin	4 min
Rinse in tap water	10 sec	Rinse in tap water	10 sec
70% alcohol	1 min	70% alcohol	1 min
95% alcohol	1 min	95% alcohol	1 min
95% alcohol	1 min	95% alcohol	1 min
100% (absolute) alcohol	3 min (dehydration)	100% (absolute) alcohol	3 min (dehydration)
100% (absolute) alcohol	3 min (dehydration)	100% (absolute) alcohol	3 min (dehydration)
50/50 alcohol/xylol	20 sec	50/50 alcohol/xylol	20 sec
Xylol	5 min (clearing)	Xylol	5 min (clearing)
Xylol	5 min (clearing)	Xylol	5 min (clearing)
Mount with DPX		Mount with DPX	

Solutions of H&E stain

Harris's haematoxylin

5 g Haematoxylin (CI: 75290)

50 ml Absolute alcohol

100 g Potassium alum (Aluminium potassium phosphate)

1000 ml Distill water

2.5 g Mercuric oxide

40 ml Glacial acetic acid

Haematoxylin was dissolved in absoulate alcohol. Potassium alum was dissolve in water by using heat. Both solution was mixed together and heated until boiling. Adding mercuric oxide in hot solution carefully (covering beck of conical flask with cotton wool). The mixture was cooled rapidly in cold water. When the mixture was completely cooled, acetic acid was added and following with filtering process. The solution stays stable for 3-4 months in dark bottles.

Scott's solution

3.5 g Sodium bicarbonate

20 g Magnesium sulphate

1000 ml Tap water

Moor's Buffered Eosin

Solution A	Solution B
11.5 ml Glacial acetic acid	16.4 g Sodium acetate
2000 ml Distilled water	2000 ml distilled water

295 ml of Solution A with 705 ml Solution B was mixed together. Then 5g Eosin yellowish (CI45380) was added into mixture with 2 crystals thymol. The final pH was adjusted to 4.98.

Solution of PAS

0.5% Periodic acid

1 g Periodic acid in 200 ml water

Schiff's reagent

1 g Basic fuchsin (CI42510)

20 ml Distilled water

2 g Sodium metabisulphate

2 ml Concentrated hydrochloric acid

2 g Activated charcoal

The basic fuchsin was heated to dissolve in distill water and then cooled the solution down. The solution was mixed with sodium metabisulphate and acid in hood then left in hood for over night. The solution was mixed with charcoal completely for 10 mins and filtered with No.1 filter paper. Storeed at 4 °C.

Mayer's haematoxylin

1 g Haematoxylin

0.2 g Sodium iodate

50 g Potassium aluminium sulphate

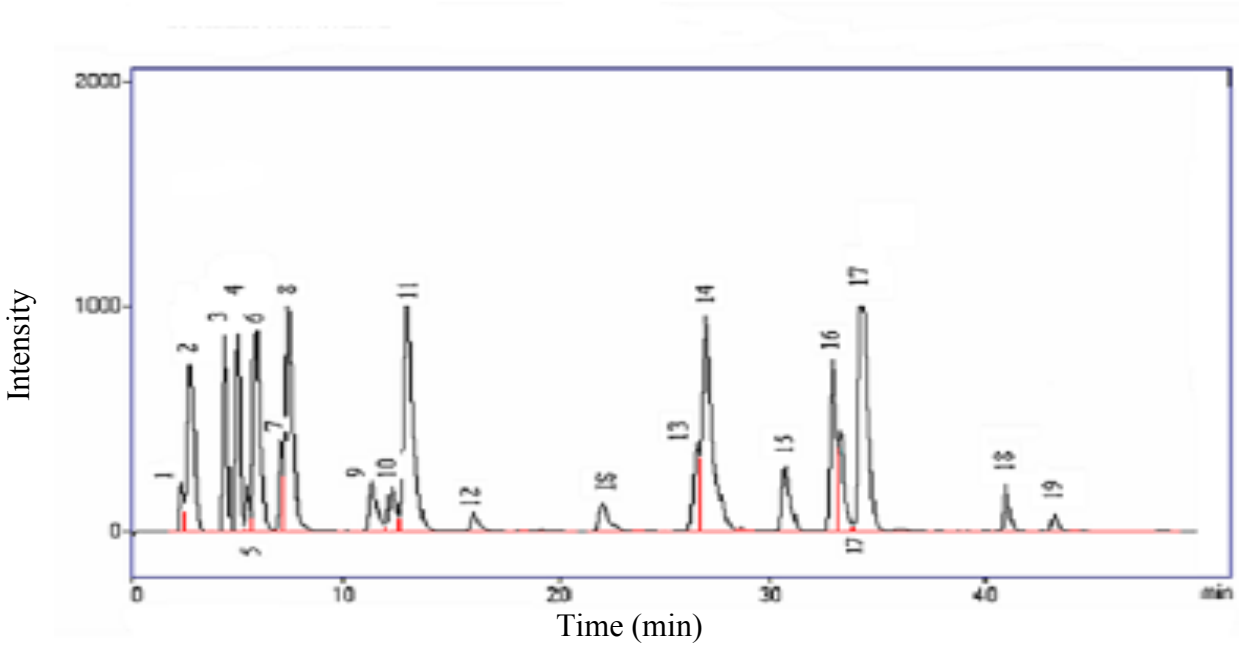
1 g Citric acid

50 g Chloral hydrate

All ingradium was disolove in 1000 ml distilled water and ripen in dark bottoles at least 7 days.

Appendix II HPLC chromatogram and gradient program

Figure II. 1. HPLC chromatogram of amino acids standard derivatized with OPA-MET.



1=Aspartic acid, 2=Glutamic acid, 3= Aspargine, 4=Serine, 5= Glutamine, 6= histidine, 7=Glycine, 8=Threonine, 9=Alanine + Taurine, 10=Arginine, 11=GABA (γ-amino butyric acid), 12=Tyrosine, IS= Internal Standard, 13=Methionine, 14=Valine, 15=Phenylalanine, 16=Isoleucine, 17=Leucine, 18=Ornithine, 19=Lysine

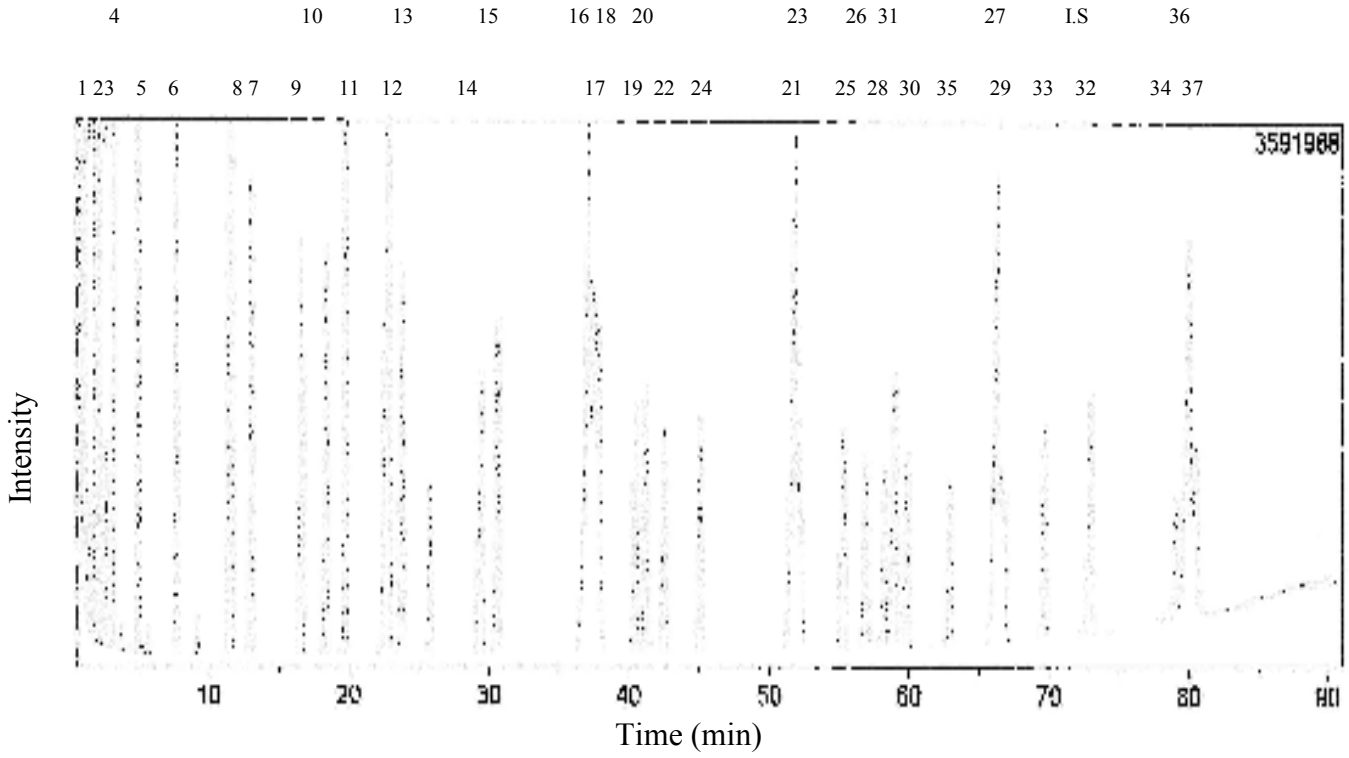
Table II. 1 Gradient solution mixing program for HPLC

Start time	Function	Gradient value (%) and total flow(ml)
0.01	total flow	0.5 ml
0.01	solution A	100%
3.00	total flow	0.5 ml
3.01	total flow	1 ml
3.01	solution A	100%
5.00	solution A	100%
5.00	solution B	0%
14.50	total flow	1.5 ml
14.50	solution A	85%
14.50	solution B	15%
19.50	solution A	85%
19.50	solution B	15%
24.50	solution A	70%
24.50	solution B	30%
29.50	total flow	1.5 ml
29.50	solution A	60%
29.50	solution B	40%
45.00	solution A	30%
45.00	solution B	70%
50.00	total flow	1.5ml
55.00	solution A	20%
55.00	solution B	80%
55.01	total flow	0.5 ml
55.01	solution A	100%
60.00	solution A	100%
75.00	STOP	

Appendix III. GC chromatogram for FAME standard (C4-C24).

Figure III. 1. GC chromatogram of FAME standard (C4-C24)(Sigma)

The numbers above peak representing fatty acid methyl ether are listed in the table below.



2	Caproic acid	(C6:0)	21	Arachidic acid	(C20:0)
3	Caprylic acid	(C8:0)	22	r-Linolenic acid	(C18:3n6)
4	Capric acid	(C10:0)	23	cis-11-Eicosenoic acid	(C20:1)
5	Undecanoic acid	(C11:0)	24	Linolenic acid	(C18:3n3)
6			25	Haneicosanoic acid	(C21:0)
7	Tridecanoic acid	(C13:0)	26		
8	Myristic acid	(C14:0)	27	Behenic acid	(C22:0)
9	Myristoleic acid	(C14:1)	28	cis-8,11,14-Eicosadlenoic acid	(C20:3n6)
10	Penladecanoic acid	(C15:0)	29	Erucic acid(C22:1n9)	
11	cis-10-Pentadecenoic acid	(C15:1)	30	cis-11,14,17-Eicosatrienoic acid	(C20:3n3)
12	Palmitic acid	(C16:0)	31		
13	Palmitoletic acid	(C16:1)	32	Tricosanoic acid	(C23:0)
14	Heptadecanoic acid	(C17:0)	33	cis-13,16-Docosahexaenoic acid	(C22:2)
15	cis-10-Heptadecanoic acid	(C17:1)	34	Lignocaric acid	(C24;0)
16	Stearic acid	(C18:0)	35	cis-5,8,11,14-Eicosapentaenoic acid	(C20:5n3)
17	Elaidic acid	(C18:1n9t)	36	Nervonic acid	(C24:1)
18	Oleic acid	(C18:1n9c)	37	cis-4,7,10,13,16,19-Docosahexaenoic acid	(C22:6n3)
19	Linolelaidic acid	(C18:2n6t)			
20	Linoleic acid	(C18:2n6c)			

Appendex IV Microalge strains description of genera and picture

(Genera descriptions were adopted and modified according to Austrid C., 1968; Humm and Wicks, 1980; Round et al., 1990)

Cynobacteriums

Family Chroococcaceae

Cos: *Coccochloris* cf. *stagnina* (Figure IV.1a)

Cell is elongate, ovoid to cylindrical, usually embedded in a gelatinous matrix to form a mass or colony in which the cells are irregularly arranged. Cell division is always in a plane at right angle to the long axis. Colonies appears as green to yellowish in color; mature cells divide into two truncate-hemispherical or truncate-ovoid daughter cells that became ovoid to elliptical and up to three times as long as broad. The sheaths at first hyaline often become yellowish with age.

It is worldwide distribution from Atlantic to North America and from Hawaii to the West Indies, especially found in brackish water and epiphyte or on solid substrata and also in the plankton collection.

Ana: *Anacystis* cf. *aeruginosa* (Figure IV.1b)

It forms microscopic or macroscopic colonies. Cell is 6-12 μm in diameter with the adjacent face flatten after division but becoming spherical before division; irregularly arrange in the colorless gelatinous matrix cell contents green in shallow water. Cosmopolitan in shallow marine or brackish water in protected place, usually attached by the sheath to solid surface.

Family Schizotrichaceae

Scc1-2: *Schizothrix* cf. *calcicola* (C. Agardh; Gomont) (Figure IV. 2a; Scc2)

Trichomes of the cell are 0.2-3.5μm in diameter and the cells are 0.2-6.0 μm long, the end wall of mature terminal cells rounded to hemispherical, not thickened. The nodes may or may not be constricted. Extracellular polysaccharides are invisible, or, if visible, soft and diffluent, or forming a distinct sheath. It is widely distributed and abundant in virtually all marine algae habitats. It survives long periods of dessication. It bores into limestone and is abundant in oyster and clam shell and produces a greenish layer in the surface.

Sca: *Schizothrix* cf. *arenaria* (Gomont) (Figure IV. 2b)

Trichomes of the cell are 1-6 μm in diameter and the cells are 2-10 μm long, the end wall of mature terminal cells distinctly conical and with a thin membrane and a broadly round end, not thickened. Cross walls are slightly distinctly constricted, without granules. Extracellular polysaccharides are invisible, or, if visible, soft and diffluent, or forming a distinct sheath. It is worldwide distributed and abundant in virtually all tidal habitats.

Family Oscillatoriaceae

Ose1-2: *Oscillatoria* cf. *erythrata* (Ehrenberg ; Kützing) (Figure IV. 3a; Ose2)

Plant is strictly planktonic and marine in the form of bundles of trichomes held together by diffluent extracellular polysaccharide. The bundles break apart as their size reaches the limit of the adhesive properties of the polysacaride, often found as 1mm in diameter and one to several millimeters long which is visible by the raw eye. Trichomes were 3-20 μm in diameter and the cell is 2-27um long. Terminal cells are cylindrical with round end; the end wall becomes thicken with age. The species breeds red-tide, with worldwide distribution.

Diatoms

Family Triceratiaceae

Bia: *Biddulphia* cf. *aurita* (Cupp) (Figure IV. 3b)

Cell are robust rectangular in girdle view, elliptical in valve view, with prominent elevations at the poles. Normally, it is seen in girdle view, it often grows in zigzag chains attached to filamentous seaweeds. A very common marine genus but taxonomically extremely confused.

The valves of it are bipolar /lanceolate to almost circular, often with wavy margins. Valve surfaces often furrowe with various thickenings, spines or ridges. Apices bearing rounded pseudoceli on low or extended elevations. Valve mantle are not well defined but extreme edge of valve often recurved and variously moulded. Areolae are large with perforate vela of the cribrum type. Simple pores also occur occasionally in the valve framework. Internally, beneaths the external indentations of the valve, there are conspicuous plain ridges (pseudosepta). One to several rimoportulae present in the cell. External tubes are often stout and surmounted by two spines. Cingula with a complete, closed valvocopula and 3-4 split couplae; areolae large, in rows; fluting of girdle corresponding to that of valve edge.

Family Entomoneidaceae

Ena1-2: *Entomoneis* cf. *alata* (Ehrenberg) (Figure IV. 4a; Ena2)

Cell is solitary, twisted about the apical axis, usually lying in girdle view and then appearing bilobate. The torsion of the cell means that valves or whole frustules can present a great variety of aspects depending on exactly how they lie relative to the observer. It is generally found as one axial (plate-like plastid) or two plastids (one on each side of the median transpical plane). It is a fairly large epipelic genus which is found in brackish marine sediments; occasionally in freshwater.

Its valves are lamceolate or linear with acute poles, often strongly compressed laterally and bearing a high, narrow keel, which becomes lower (and may disappear) in the centre of the valve, and also decreases in height towards the poles. Discrete mantles usually absent. Outside of valve, the cells sometimes bear warts. Their valve margins and raphe-sternum sometimes appear with longitudinal ribbing. Striae is usually biseriate or multiseriate, containing small round poroids occluded by hymens; or the whole stria consisting of little more than a delicate, porous siliceous membrane (in which case the perforations are usually still in two lines, next to the transpical costae). Raphe system is fibulate, or the sides of the keel fused beneath the raphe so that the subraphe canal is connected to the rest of the cell lumen only near the central and polar raphe endings. In fibulate forms, fibulae may occur at many levels beneath the raphe, especially where the keel is high; the fibrulae are short, bar-like struts and are borne on the transapical costae. Central raphe endings (internal and external) and external polar ending are all similar inlooking: straight, and not or only slightly expanded. The external raphe fissure ends are very close to each pole. Girdle bands are numerous, open and porous.

The more familiar family name *Amphiprora* was abandoned in favour of *Entomoneis* .

Family Naviculaceae

Nam : *Navicula* cf. *minuscule* (Figure IV. 4b)

Naa : *Navicula* cf. *atomoides* (Figure IV. 5a)

Naf 1-2*:* *Navicula* cf. *falaisensis* (Figure IV. 5b; Naf2)

Nap: *Navicula* cf. *perminuta* (Figure IV. 6a)

Cells are solitary in naviculoid. Most species lie in valve view but a few are strongly compressed laterally and lie in girdle view. Two girdle-appressed plastids, one on either side of the apical plane, each contains an elongate rod-like pyrenoid. The species were found epipeliily in freshwater or marine environments.

The valves of cell are lanceolate to linear, with blunt rostrate or capitate apices. Valve face is flat or curved, usually curving gently into the mantles. A short conopeum is sometimes present: striae, uniseriate or rarely bi-seriate, which are closed by hymens at their inner apertures. The periods of adjacent striae are aligned with each other, so that straight or gently curving longitudinal striations are usually visible in the LM. Striae interrupted by lateral sterna in a few species. Raphe-sternum appear thickened, especially on the primary side, so that the internal fissures open laterally, except at centre and poles; the primary side usually bears in addition an accessory rib running the whole length of the valve internally, parallel to the raphe. Central internal raphe endings straight and unexpanded, lying in a small oval nodule, sometimes the central nodule; rarely a double helicatoglossa is formed. External central endings are simple, expanded into pores or hooked. Girdle composed of several open, usually plain bands.

Family Bacillaraceae

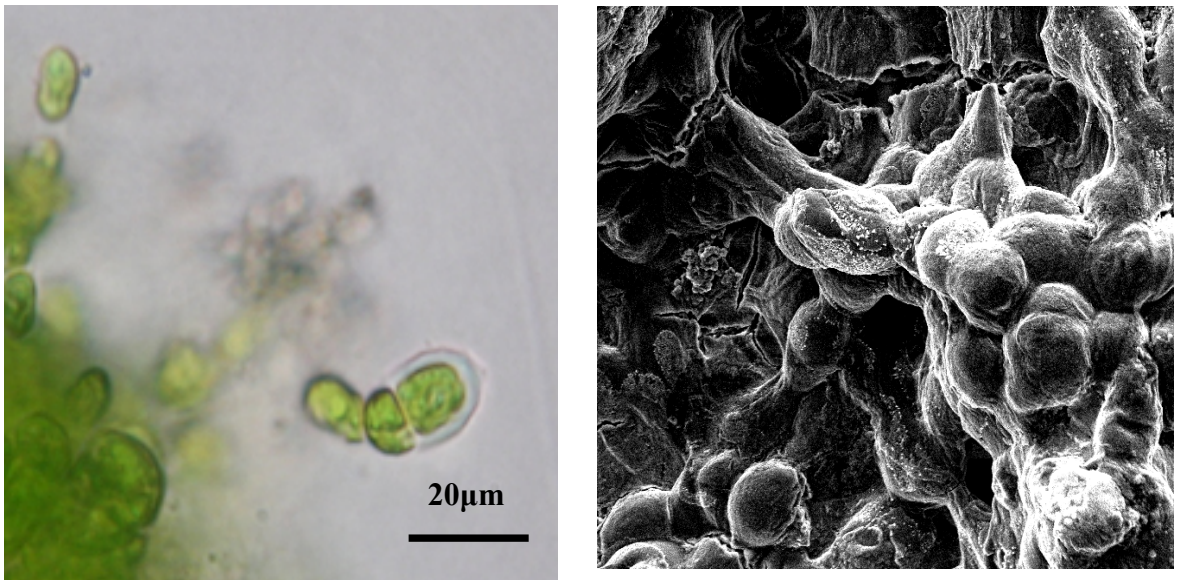
Cya 1-2: *Cylindrotheca* cf. *acicularis* (Heurck) (Figure IV. 6b; Cya1)

Cyc1-3: *Cylindrotheca* cf. *closterium* (Heurck) (Figure IV. 7a; Cyc2)

Cell is solitary, needle-like and straight in shape. Frustules of cell are usually strongly twisted about the apical axis so that valve and girdle describe a spiral course around the cell; consequently the cells rotate as they move through the sediment. Plastides are two to many, which are plate-like or discoid. It is a small genus in benthic diatoms but widely distributed worldwide. Most species are permanently epipelic. Their valves are long and narrow and only lightly or partially silicified, often reduced to strips of material bordering the raphe. Striae are irregular if present. Their delicate sinuous costae extend out from the raphe-sterum and these are separated by area of small pores. Raphe is subtended by numerous thin, rib-like fibulae, which are relatively heavily silicified. Central raphe ending is present or absent, in which case the raphe is continuous. When present, the endings are straight and very slightly expand. Polar ending are simple. Girdle band is narrow and numerous, consisting of light silicified plain strips.

Figure IV. 1. (a) Left: Light microscope image of *Coccochloris* cf. *stagnina* Olympus BX41 400X. Right: EM image, 3000X, HV5.0KV, WD 10.1mm, Spot 2.0. (b) Left: Light microscope image, *Anacystis* cf. *aeruginosa* Olympus BX41 400X. Right: EM image, 1600X, HV5.0KV, WD 10.1mm, Spot 2.0.

(a)



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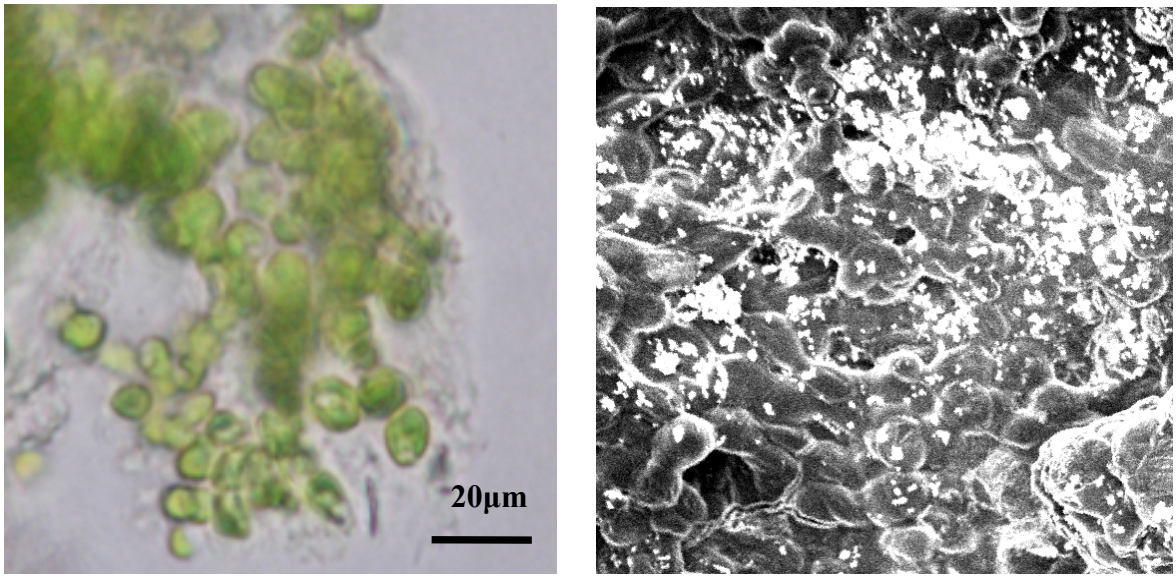
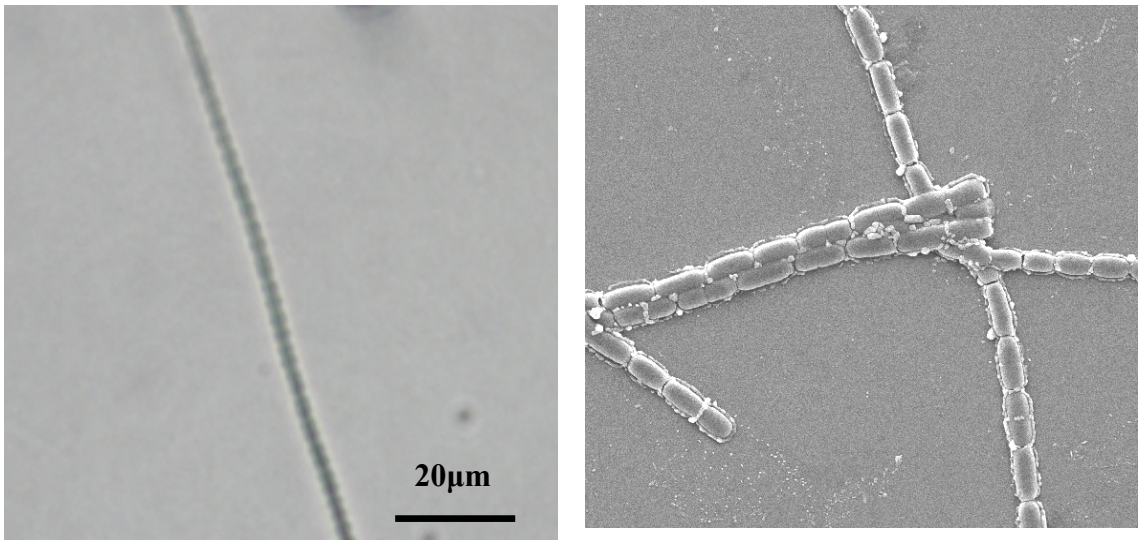


Figure IV. 2. (a) Left: Light microscope image of *Schizothrix* cf. *calcicola* Olympus BX41 400X. Right: SEM image, 6000X, HV5.0KV, WD 10.3mm, Spot 2.0. (b) Left: Light microscope image of *Schizothrix* cf. *calcicola* Olympus BX41 400X. Right: SEM image, 12000X, HV5.0KV, WD 10.3mm, Spot 2.0

(a)



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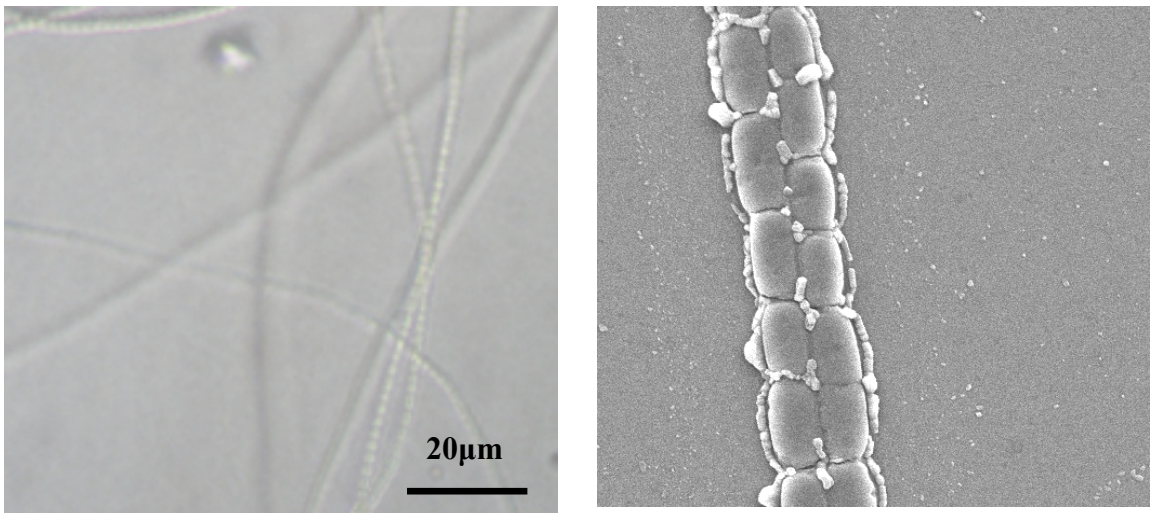
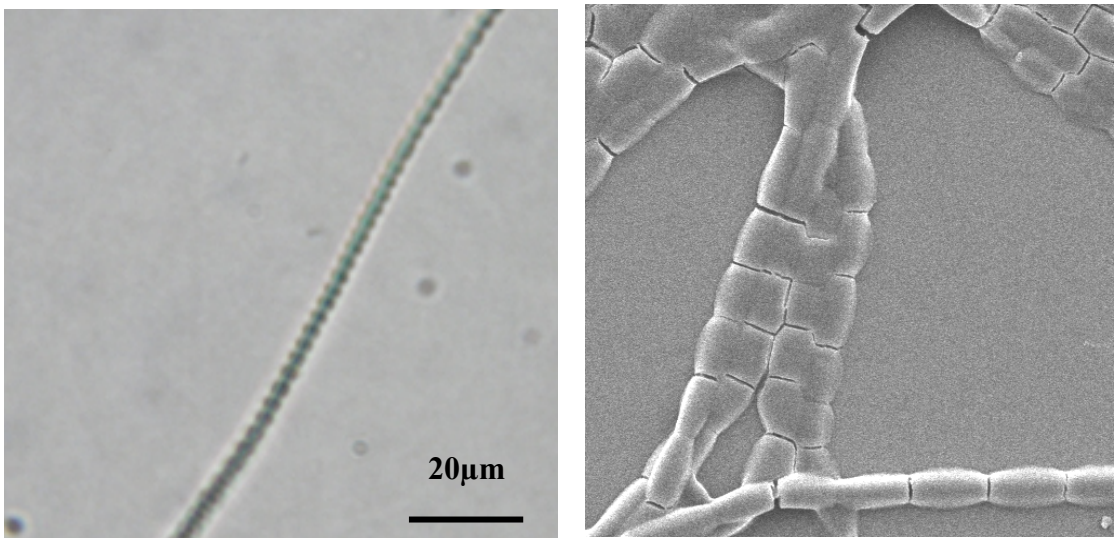


Figure IV. 3. (a) Left: Light microscope image of *Oscillatoria* cf. *erythrata* Olympus BX41 400X. Right: SEM image, 6000X, HV5.0KV, WD 10.3mm, Spot 2.0. (b)Left: Light microscope image of *Biddulphia* cf. *aurita* Olympus BX41 400X. Right: SEM image, 4000X, HV5.0KV, WD 9.9mm, Spot 2.0.

(a)



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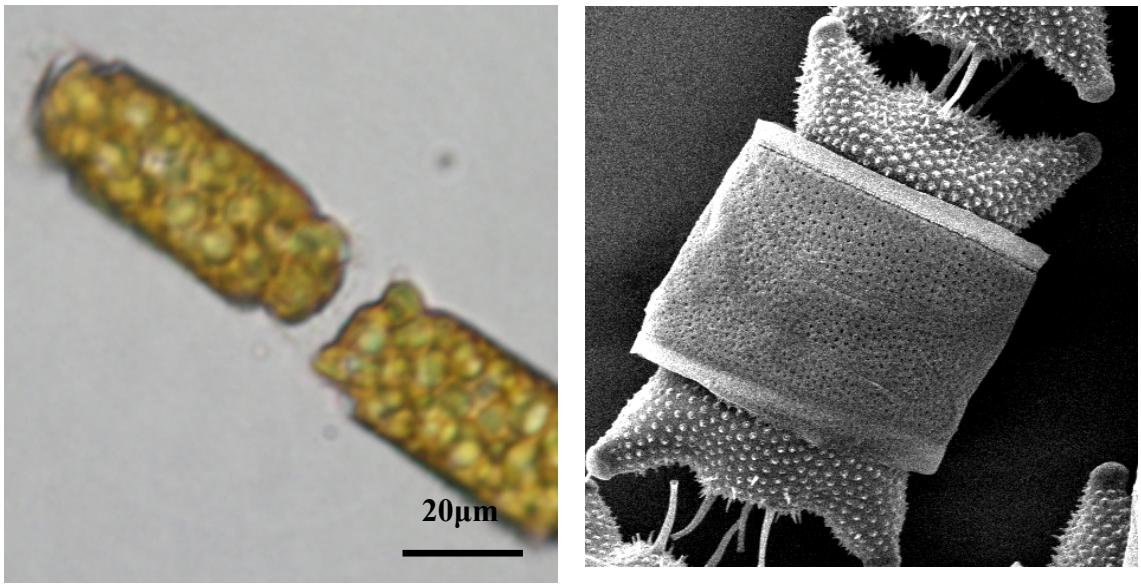
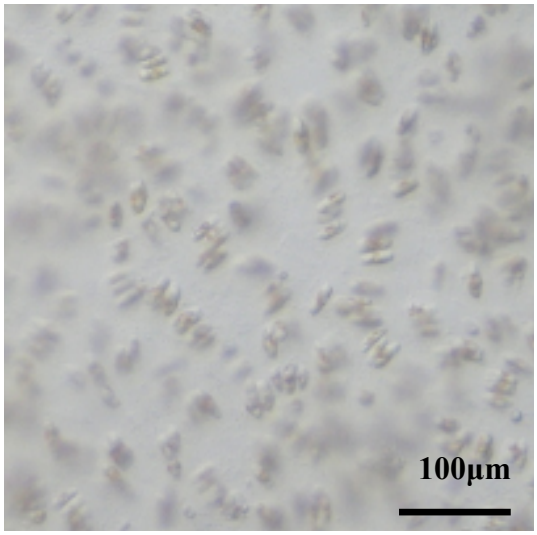
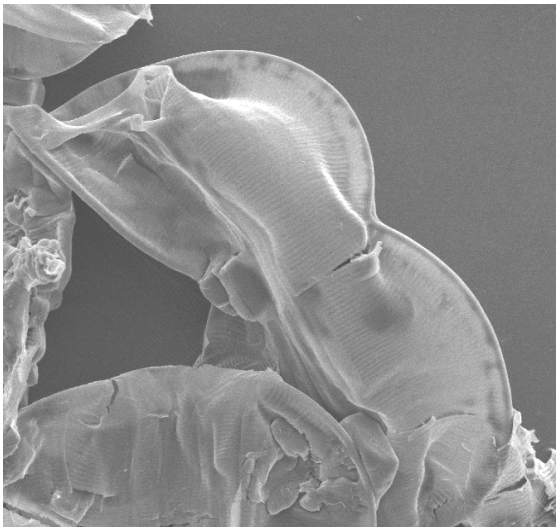


Figure IV. 4. (a) Left: Light microscope image of *Entomoneis* cf. *alata* Olympus BX41 100X. Right: SEM image, 6000X, HV5.0KV, WD 10.9mm, Spot 2.0. (b) Left: Light microscope image of *Navicula* cf. *minuscula* Olympus BX41 400X. Right: SEM image, 15000X, HV5.0KV, WD 11.2mm, Spot 2.0.

(a)





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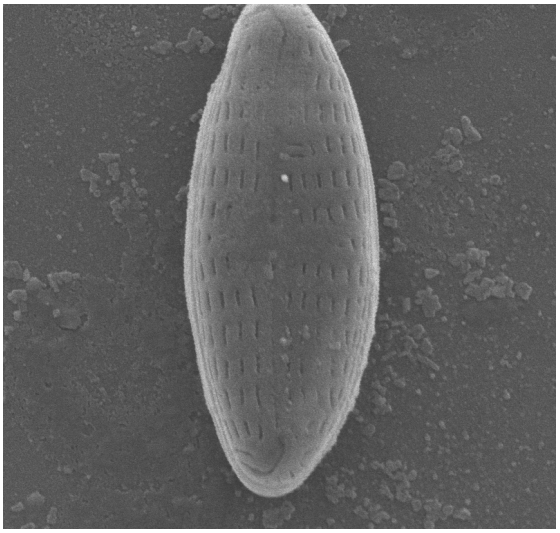
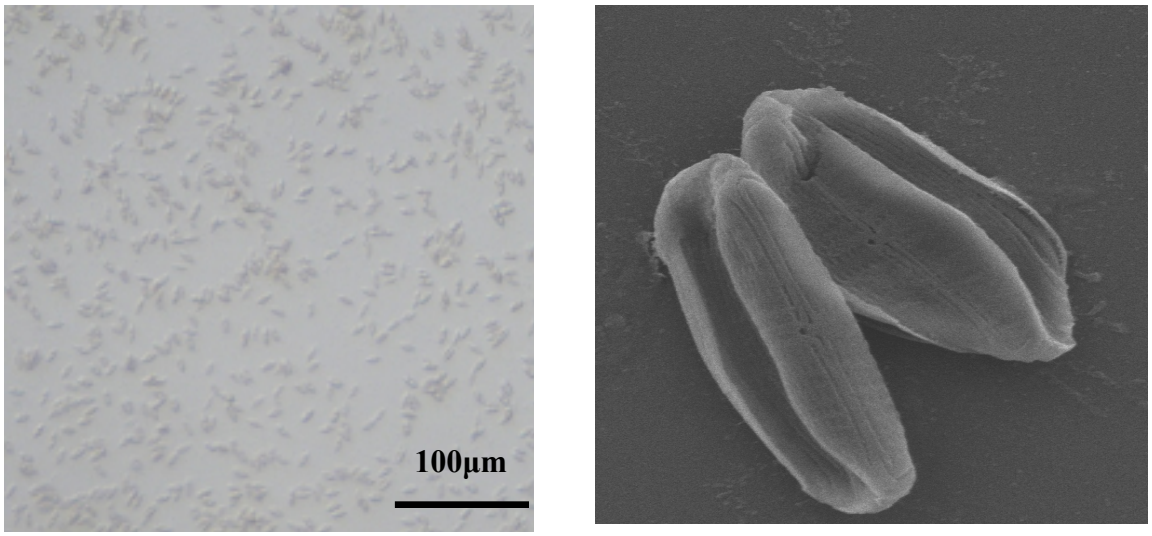


Figure IV. 5. (a) Left: light microscope image of *Navicula* cf. *atomoides* Olympus BX41 100X. Right: SEM image, 24000X, HV5.0KV, WD 10.0mm, Spot 2.0. (b) Left: light microscope image of *Navicula* cf. *falaisensis* Olympus BX41 100X. Right: SEM image, 8000X, HV5.0KV, WD 11.2mm, Spot 2.0.

(a)



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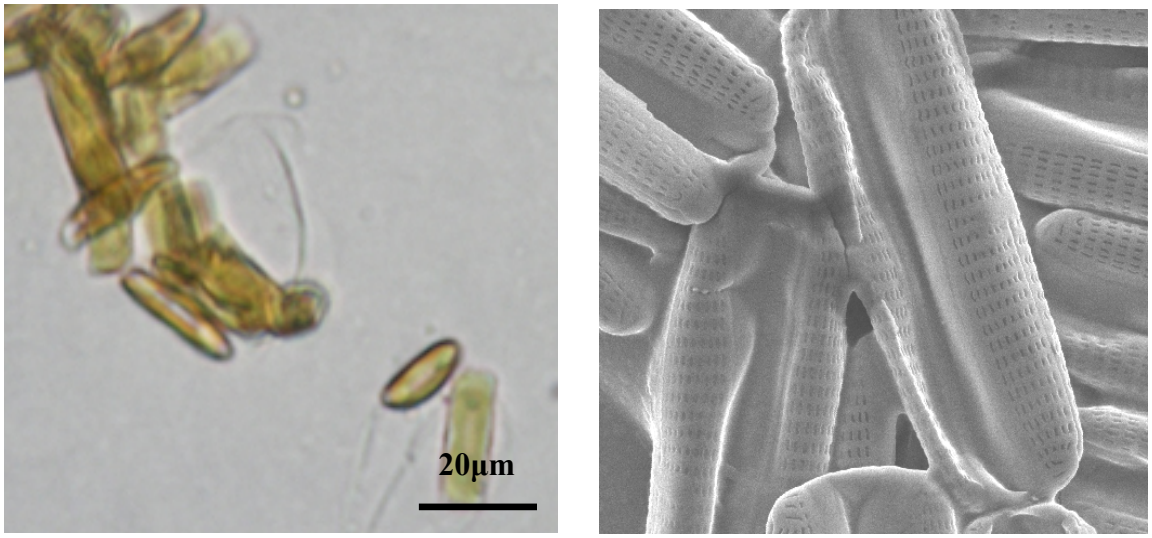
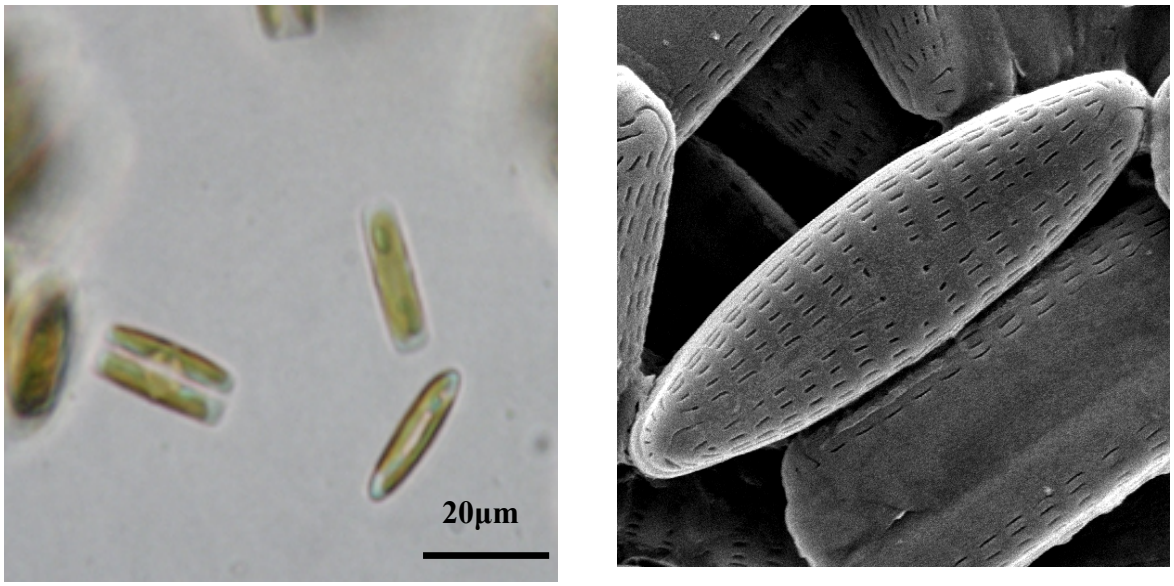


Figure IV. 6. (a) Left: light microscope image of *Navicula* cf. *perminuta* Olympus BX41 400X. Right: SEM image, 8000X, HV5.0KV, WD 10.4mm, Spot 2.0. (b) Left: light microscope image of *Cylindrotheca* cf. *acicularis* Olympus BX41 200X. Right: SEM image, 3000X, HV5.0KV, WD 10.9mm, Spot 2.0.

(a)



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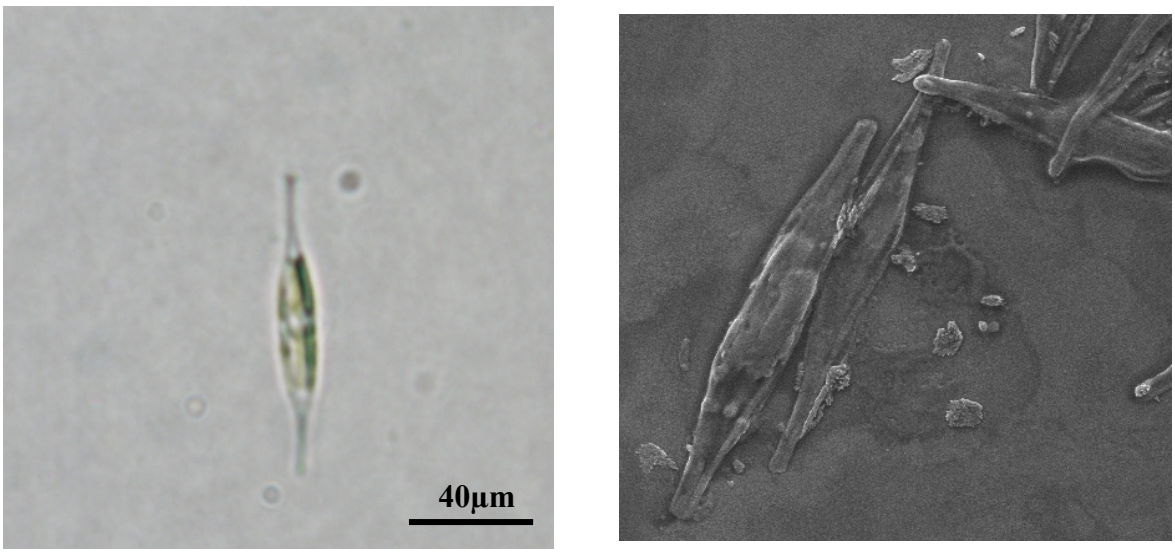

Figure IV. 7. Left: light microscope image of *Cylindrotheca* cf. *closterium* Olympus BX41 200X. Right: SEM image of, 8000X, HV5.0KV, WD 10.0mm, Spot 2.