The Effect of Diet on Intestinal Structure and Function in the Abalone (*Haliotis laevegata*).

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A THESIS SUBMITTED TO THE UNIVERSITY OF ADELAIDE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

November 2001.

Final Submission
Nov- 2005

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Abbreviations:

$A	Australian dollars
$US	United States dollars
ANFs	Anti-nutrient factors.
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
FRDC	Fisheries, Research and Development Corporation
LAP	Leucine amino-peptidase
MUFA	Monounsaturated fatty acids
NSPs	Non-starch polysaccharides
P_c	Critical pressure
PUFA	Polyunsaturated fatty acids
SA	Specific activity
SA:TA	Specific activity: total activity ratio
SARDI	South Australian Research and Development Institute
SFA	Saturated fatty acids
TA	Total activity
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TRIS	Tris(hydroxymethyl)aminomethane

Chapter 3
Experimental groups

WRA	Wild reared abalone
CRA	Commercially reared abalone
CRA1	The intestine of commercially reared abalone fed on commercial diet 1
CRA2	The intestine of commercially reared abalone fed on commercial diet 2
CD1	Commercial diet 1
CD2	Commercial diet 2
Wild	Wild reared abalone intestine

Chapter 4
Experimental groups

S	The intestine of abalone fed the raw soyflour diet
SP	The intestine of abalone fed the raw soyflour + phytase
SH	The intestine of abalone fed the heated (autoclaved) soyflour
L	The intestine of abalone fed the raw lupins diet
LP	The intestine of abalone fed the raw lupins + phytase
LH	The intestine of abalone fed the heated (autoclaved) lupins
V	The intestine of abalone fed the raw vetch diet
VP	The intestine of abalone fed the raw vetch + phytase
VH	The intestine of abalone fed the heated (autoclaved) vetch
P	The intestine of abalone fed the raw peas diet
PP	The intestine of abalone fed the raw peas + phytase
PH	The intestine of abalone fed the heated (autoclaved) peas
B	The intestine of abalone fed the raw beans diet
BP	The intestine of abalone fed the raw beans + phytase
BH	The intestine of abalone fed the heated (autoclaved) beans

Chapter 5
Experimental groups

S	The intestine of abalone fed the raw soyflour diet
SR	The intestine of abalone fed on a diet containing soyflour with kaolin substitution for portion of the pregelled starch
L	The intestine of abalone fed the raw lupins diet
LR	The intestine of abalone fed on a diet containing lupins with kaolin substitution for portion of the pregelled starch
ST	The intestine of abalone fed the pregelled starch diet
STR	The intestine of abalone fed on a diet containing pregelled starch with kaolin substitution for portion of the pregelled starch
V	The intestine of abalone fed the raw vetch diet
VRI	The intestine of abalone fed on a diet with a reduced inclusion level of vetch, maintained isonitrogenous by a substitution of soyflour, and an increased inclusion of pregelled starch
P	The intestine of abalone fed the raw peas diet
PRI	The intestine of abalone fed on a diet with a reduced inclusion level of peas, maintained isonitrogenous by a substitution of soyflour, and an increased inclusion of pregelled starch
B	The intestine of abalone fed the raw beans diet
BRI	The intestine of abalone fed on a diet with a reduced inclusion level of beans, maintained isonitrogenous by a substitution of soyflour, and an increased inclusion of pregelled starch

Chapter 6
Experimental groups

FRDC	The intestine of abalone fed the FRDC diet
ST5	The intestine of abalone fed the FRDC diet with inclusion of 5% pregelled starch
B5	The intestine of abalone fed the FRDC diet with inclusion of 5% bentonite
S5	The intestine of abalone fed the FRDC diet with inclusion of 5% sand
DE5	The intestine of abalone fed the FRDC diet with inclusion of 5% diatomaceous earth
K5	The intestine of abalone fed the FRDC diet with inclusion of 5% kaolin
K10	The intestine of abalone fed the FRDC diet with inclusion of 10% kaolin
K15	The intestine of abalone fed the FRDC diet with inclusion of 15% kaolin
K20	The intestine of abalone fed the FRDC diet with inclusion of 20% kaolin

Chapter7
Experimental groups

WRA	Wild reared abalone
CRA	Commercially reared abalone
P	The intestine of abalone fed a diet of raw peas
PH	The intestine of abalone fed a diet with the dry heat treatment of peas
L	The intestine of abalone fed a diet of raw lupins
LH	The intestine of abalone fed a diet with the autoclaved heat treatment of lupins
%CAP	Percentage of the control of the alkaline phosphatase activity

Chapter 8

Experimental groups

S	The intestine of abalone fed the raw soyflour diet
SP	The intestine of abalone fed the raw soyflour + phytase
SH	The intestine of abalone fed the heated (autoclaved) soyflour
L	The intestine of abalone fed the raw lupins diet
LP	The intestine of abalone fed the raw lupins + phytase
LH	The intestine of abalone fed the heated (autoclaved) lupins
V	The intestine of abalone fed the raw vetch diet
VP	The intestine of abalone fed the raw vetch + phytase
VH	The intestine of abalone fed the heated (autoclaved) vetch
P	The intestine of abalone fed the raw peas diet
PP	The intestine of abalone fed the raw peas + phytase
PH	The intestine of abalone fed the heated (autoclaved) peas
B	The intestine of abalone fed the raw beans diet
BP	The intestine of abalone fed the raw beans + phytase
BH	The intestine of abalone fed the heated (autoclaved) beans
PDH	The intestine of abalone fed on a diet of peas with a dry heat treatment
L5H	The intestine of abalone fed a diet of lupins with a 5-minute 120°C (autoclaved) heat treatment

Abstract:

Introduction:

Feed is the largest operating cost in abalone aquaculture. Current research is looking at locally grown legumes as potential protein replacements for imported, high cost, fishmeal and soyflour. Legume consumption may be inappropriate for abalone due to the wide variety of antinutrient factors (ANFs), which can negatively impact on intestinal structure and function, hence reducing feed utilisation by abalone. The endeavour of this study was to elucidate the nature; possible mode of action and effects, and known methods for ANFs amelioration on the intestinal integrity of commercially reared (CRA) abalone *Haliotis laevegata*

Intestinal Morphology.

The predominant morphological change in the CRA, fed terrestrial protein sources, in comparison to wild reared abalone (WRA), was the increase in the mucus cover over the epithelium. Changes at the cellular level included, increased numbers of secretory cells; cell nuclei were centrally located, increased non-vacuolated cells and a profound infiltration of haemocytes in the epithelium. The response seen is typical of an inflammatory response and is most likely due to ANFs, particularly antigenic proteins.

Functional Characteristics.

Final digestive enzymes common to most mammals and fish were found in the intestinal apical membrane. Of the carbohydrases tested maltase had the greatest activity while β-galactosidase indicated association with intestinal mucus levels. In abalone groups fed the commercial, soyflour and legume diets the intestinal enzymes activities suggested inhibition of these enzymes by components of the diet. Several possible contributing factors and treatments were tested including,

Enzyme and Heat Treatment.

Phytase addition to diets needs to be assessed on an individual basis, however, abalone, when fed legumes with high phytate content, did benefit from this treatment. The heat treatment of soyflour and the legumes had resulted in a maillard reaction seen by the reduction in protease activity in all groups.

Ingredients.

The capacity of abalone to digest the complex storage starches of legumes (amylose) was low and carbohydrase activity increased with diets higher in amylopectin levels. Pregelled starch inclusion up to 25% in diets increases carbohydrate activity. The inclusion level of legumes varied due to the protein weight content, requiring high levels of peas, beans and vetch to provide isonitrogenous diets. A reduction in inclusion levels increased enzyme activity and may be related to a reduction in the ANFs present in the diet. The inclusions of the inert fillers, bentonite (5%) and kaolin (10%) provided a stimulatory effect on the intestinal enzymes of abalone.

Mechanisms.

The cell agglutination pattern suggests that diet or rearing regime changes the glycosylation pattern of the intestinal cell membranes. A feeding trial, with diet selection based on agglutination patterns, correlated with predicted results. The ability to test raw and treated feed sources without the need for lengthy feed and growth trials may prove beneficial.

Dietary Effects.

The lipid composition of the foot was changed by each legume diet and treatments. No direct evidence was found that implied ANFs from legumes were effecting fatty acid chain elongation and desaturation. Diets that were not fully digested were excreted into the farm water systems affecting water quality. A reduction in the effects of ANFs on the intestine, by diet treatments, may improve digestion and hence water quality. The properties of non-nutritive fillers, such as ion exchange and absorption, may benefit abalone farms even if no intestinal function improvement were found.

Conclusions.

The use of legumes in abalone diets may produce adverse effects on digestive structure and function however some of these effects can be removed by dietary treatments. More information is required on ANFs in legumes before their full potential in abalone diets can be realised. There are similarities with other animals and fish as to the effects of ANFs seen in abalone and as such the plethora of research studies on ANFs should be used to expedite research on abalone diets.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the Adelaide University Library, being available for loan and photocopying.

Date: 9/11/2005.

Chapter 1

Review

1. Review.

1.1. General Introduction to Abalone Aquaculture

Abalone are marine snails and belong to the Phylum Mollusca, Class Gastropoda, Subclass Prosobrancia, Order Archaeogastropoda, Family Haliotidae. Linnaeus gave the generic name *Haliotis*, meaning "sea ear," in 1758 (Bevelander, 1988). There are approximately 100 species of abalone found world wide in temperate waters ranging from low tide to depths exceeding 30metres. Abalone are often found attached to rocks and among seaweed (algae), which they use as their main source of food. Abalone have been a food source since ancient times with the shells used in jewellery and for other decorative purposes. In Australia *Haliotis rubra*, (blacklip) and *Haliotis laevigata* (greenlip) are used as food sources. The common names blacklip and greenlip are from the colour of the edge of the foot, which are distinctly black or green.

In Australia the aquaculture of abalone first commenced in approximately 1980 in South Australia and in Tasmania through research on spawning blacklip and greenlip abalone. By comparison, abalone aquaculture is over 50 years old in Japan and China. The impetus for developing abalone aquaculture in Asia was a decline in their wild harvest fisheries due to over exploitation. The stock of wild abalone in Japanese waters is reliant on the release of 30 million hatchery-produced larvae to enhance a fishery, which has had a steady decline for 20 years (Rudd, 1994). In Australia fisheries management has partially controlled the wild harvest at sustainable levels, however the Australian catch had declined during the 1980's (Kailola *et al.*, 1993). China and Taiwan are the main producers by aquaculture in Asia, however China is reaching the limits of sites suitable for development. China also has considerable problems with typhoons that frequently cause extensive damage to farms. This means Chinese farmers need to gain a fast return from these sites to be viable. Taiwan's farmed product is tropical abalone that has poor market acceptance due to its tissue quality (Vanderpeer *et al.*, 1999) and Taiwan also has water quality problems. Both China and Taiwan import abalone, as their internal demand cannot be met by internal production. Oakes and Ponte (1996) believed that a lucrative Asian market niche existed for high quality abalone cultured in western countries despite their labour costs.

Rising world abalone prices in the 1980's have made abalone aquaculture in Australia a viable proposition. In 1994 Australia supplied over 40% of the worlds wild-harvested abalone and its two commercial species have significantly favourable price margins over abalone species

harvested from New Zealand, Chile and Europe. In 1994 prices for Australian abalone reached record high prices of $US38-45/kg whole weight for that era. The markets for aquaculturally produced and wild abalone products are quite different. Australia's wild abalone has restricted markets due to minimum size limits imposed on capture (113 mm). The aquaculture product can produce abalone of any size. The world market for abalone produced by aquaculture is generally characterized by size (50-100 mm), is sold mainly live and commands a higher price than live wild abalone. As an example, in the 1994 Hong Kong market Australian live wild abalone of size 120-145 mm (350-400 g) received $US32/kg whereas Australian live aquaculturally produced abalone of size 60-80 mm (75-90 g) fetched $US45-50/kg (Rudd, 1994). In terms of canned abalone $US price in the world market, Australian abalone currently sits in third place behind Mexico and South Africa (Johnston, 2001). The world abalone market is an open market and is fairly volatile with price movements changing rapidly due to supply and demand. Currently (2001) Australian exporters fetch $US420/carton for canned abalone, however this has been as high as $US530 in 1997 (Johnston, 2001).

In the late 1980's and early 1990's commercial aquaculture farms in Australia had business plans that based their internal rate of return on a price for abalone of between $US25-30/kg. They used a figure of 4-5 years production for abalone to reach market size of 80-100 mm (Hone and Maguire, 1996). To allow the Australian abalone aquaculture industries to meet expanding market demands in Asia and remain competitive, research during the 1990's focused on reducing production costs and time to market size. The main research areas were nutrition, tank design, water quality and health.

1.2 The Farmed Abalone Industry in Australia. Production Factors other than Nutrition.

1.2.1 Tank Design.

A number of alternative designs were trialed by industry during the early 1990's with various problems occurring, especially with waste removal and water quality (Hindrum, 1994; Maguire, 1994; Morrison, 1994). Most early tanks were fibreglass with false bottoms of mesh. This allowed particulate wastes to settle to the bottom for subsequent removal by siphon or flushing. Several disadvantages were shown. If water flow stopped during summer the water rapidly heated up to temperatures that could kill the abalone (Hindrum, 1994). The use of shelters within tanks (PVC pipe halved) interfered with water flow and caused build up of faecal waste

(Hindrum, 1994; Morrison, 1994). Dissolved oxygen levels were found limiting to production in tanks where faecal and feed waste settled rather than remaining suspended (Maguire, 1994), and high ammonia concentrations occurred from decay of organic matter (faeces, food) if not removed efficiently (Hindrum, 1994; Maguire, 1994). Tanks were deep sided, which proved to be labour intensive to clean because water had to be removed before the build up of fouling on the sub floors could be removed. This was a daily occurrence to remove excess food (Maguire *et al.*, 1996) and abalone were subjected to extended periods of aerial exposure (emersion) during this drainage procedure. Whang and Chung, (1977) found that juvenile *Haliotis discus* could survive several hours emersion if kept moist. Abalone can use anaerobic pathways during emersion (Wells and Baldwin, 1995) however changes in respiration rate are evident after periods of emersion (Edwards, 1996). All of the above problems were responsible for a depression in growth (Wells and Baldwin, 1995). From these preliminary designs, the key to maintaining optimal environmental conditions is to ensure that as the animal produces wastes, the tank water dynamics are such that they are quickly washed away along with unused feedstuffs from the animals.

A move to "raceway" style tanks of low volumetric capacity has alleviated many of these problems in that waste is removed with a sufficient rate of water velocity. This water velocity also has the effect of stimulating feeding. Wild greenlip abalone in their natural conditions when exposed to high flow rates, adopt a distinctive feeding posture whereby they raise the front of their shell and catch drift weed with their foot instead of grazing on benthic algae (Shepard, 1973). Aquaculture abalone adopt this feeding posture in high flow rates. Flow rates have been shown to affect growth. As flow rate increases from 2-20L/min, the removal of wastes from the internal cavity of the shell and around the abalone increases, preventing the localized build up of detrimental wastes. At high flow rates (20L/min) abalone are only able to react quickly enough to catch food as it moves past them when abalone density is high enough to trap food against their shells (Higham *et al.*, 1998). Flow rates of 3L/min have been found to be a compromise between space utilization by abalone and average growth rate (Higham *et al.*, 1998).

1.2.2. Water Quality.

Water is pumped directly from the sea for all land-based farms and the water is recirculated on the farm to reduce costs. Abalone may be therefore exposed to water quality conditions that vary from optimal to levels that may reduce growth. Detrimental conditions may include, excess

nitrogenous wastes, low dissolved oxygen levels, high temperatures, changes in pH and salinity. Nitrification, from decay of organic material, will cause pH levels in tanks to decline (Harris *et al.*, 1998a). In aquatic animals pH values outside a range of pH 5-9 are often lethal (Randall, 1991). Harris *et al.*, (1998a) found in greenlip abalone EC_5 (effective concentration for 5% growth reduction) value for specific growth rate to be pH 8.77 and an EC_{50} (median growth reduction) value pH 7.39. They also found both an increase and decrease in pH from pH 7.76 decreased food consumption.

Ammonia is toxic to aquatic animals, causing damage and behavioural change at the cellular, organ and whole-body level. Alterations to reproductive capability, histological structure, and osmoregulation, growth and disease susceptibility have all been documented as effects of ammonia toxicity (Colt and Armstrong, 1981). Ammonia is a naturally-occurring product of metabolic degradation of protein (Russo and Thurston, 1991) and excreted nitrogenous product (Colt and Armstrong, 1981) in aquatic systems and is a common component of the aquaculture environment (Tomasso, 1996). Harris *et al.*, (1998b) found that as unionised ammonia increased (0.006- 0.188 mgN/L) the specific growth rate of both length and width decreased. They also found that food consumption decreased. They found an EC_{50} (50% survival) for survival at 0.188 mg N/L.

In some abalone production systems, where uneaten food and decaying wastes are only removed intermittently, the biological oxygen demand is high. *Haliotis laevegata* are known to be sensitive to low dissolved oxygen levels with decreased specific growth rate EC_5 and EC_{50} at saturation levels 96% and 77% respectively (Harris *et al.*, 1999). Abalone appear to be oxygen regulators in that they consume oxygen independent of external concentration down to a specific level (P_c critical pressure), below which they behave as oxygen conformers (Jan and Chang, 1983). The region above Pc is referred to as the zone of respiratory independence and that below respiratory dependence (Jan and Chang, 1983). Consumption by oxygen conformers is dependent on external concentrations. Research has shown that growth increases may occur for abalone when concentration is 115% saturation (Leitman, 1992), while oxygen levels in excess of 115% are known to increase the incidence of gas bubble disease, bacterial infection and subsequent mortality (Elston and Lockwood, 1983; Elston, 1983). However, Coote *et al.*, (1996) found length growth rates of 0.59% day $^{-1}$ for greenlip abalone when exposed to an oxygen level of 120% saturation.

The temperature of the water, in land based farms, is not kept constant due to the high cost in refrigeration and heating. In South Australia the seawater temperature can range between 16°C-24°C. Optimum temperatures for growth in greenlip abalone have been found to be 18.3°C (Gilroy and Edwards, 1998). In summer months in South Australia high seawater temperature has several effects. The stomach of the abalone swells to such an extent it forms a blister between the foot and the shell. In extreme cases the abalone float to the surface or die. This swelling has been noted in other areas of Australia and in Japan (Fleming *et al.*, 1996). It is thought the cause of the blisters is the fermentation of the protein sources of the feed in the gut (Fleming *et al.*, 1996). In *Haliotis midae* both feed consumption and growth decreased significantly at temperatures above 20°C with the animals showing visible signs of stress at temperatures between 22-24°C (Britz *et al.*, 1997). In Australia when abalone show indications of stress due to temperature farmers stop feeding for up to one month. The consequence of this on the growth rate, the effect on the digestive tract of the abalone and how well they respond once feeding is resumed has not been investigated. The rate of digestion has been shown to decrease after periods of starvation in *Haliotis rubra* and decreases further with increased periods of starvation (Foale and Day, 1992). Increased temperature also causes a marked increase in bacterial growth within the tank, the substrates for the bacteria being uneaten food or faecal waste. The build up of bacteria slows food consumption by the abalone.

1.2.3 Stocking Rates.

Optimal commercial stocking densities were found by trials involving stocking rates ranging between 25% and 150% of the area of the tank in which the abalone choose to reside (Moore and Hone, 1994). At increases in density above 75% effective surface area, growth rates were suppressed. Abalone when stocked at high densities fight for space, which causes shells to be broken. The broken shells can cut the foot of neighbours resulting in the death of the animal, as clotting of hemolymph is poor in abalone. Optimal yield (weight), which occurs at high stocking rates, is not a valid basis for such high stocking rates as the time to reach market will be slower. Therefore, in calculating the stocking density that offers the best economic return, it is important to balance the fixed capital costs with the variable input costs of running the system for a longer period. Most economic analyses tend to suggest that reducing the production cycle time provides the greatest economic return (Moore and Hone, 1994).

1.2.4. Translocation of Abalone.

Among commercial enterprises it is necessary to grade abalone into size, which means moving abalone into different tanks. The use of the anaesthetic benzocaine to enable translocation may markedly suppress growth in the short-term (up to 1 month) (Dunstan *et al.*, 1998). Losses can occur through the use of flat knives to dislocate the abalone from the tank, which can damage the foot, resulting in mortalities.

1.2.5. Parasitic Infestation.

Following the commencement of sea based abalone culture in Tasmania and land-based farms in South Australia high levels of mudworm were found in abalone. The term "mudworm" refers to a group of polychaete worms that generally start to burrow at the apex or the edges of the shell leaving a tunnel shaped raised 'blister' within the shell. A high level of shell damage is often seen. Examination of the inside surface area of abalone shell in infected animals show blisters cover from <5% to 50% of the shell surface area (Lleonart and Handlinger, 1997). In severe infestations, the mudworm can kill the abalone, although the exact mechanism of this is not known (Lleonart and Handlinger, 1997). Treatment by air-drying the abalone significantly reduces infestation (Lleonart, 1999) although the stress of emersion, as mentioned before, does have the effect of reducing growth.

The production problems described in 1.2.1 to 1.2.5 above have all had an impact on growth however most have been overcome through improved farming practices, tank design and with water quality being a particularly important component. With these advances, the time to market size (80-100mm) has been reduced by 1-2 years from 4-5 years (Hone and Maguire, 1996).

Concurrent with the above production factors there has been considerable effort placed on nutrition research. In Australia, to achieve industry requirements of 70-100μ m/day growth rates, feed conversion ratios of 1.3-1.5 at a feed cost of $AUS 2.00-3.50/kg a suitable artificial feed was needed (Fleming *et al.*, 1999). To understand the nutritional needs of abalone several parameters need to be known such as feeding behaviour, growth conditions, the natural diet of the abalone and what are their protein, energy and mineral requirements.

1.3. Feeding Behaviour.

Feed is the largest operating cost in intensive aquaculture. There have been a number of studies on factors influencing the feeding behaviour and gastric evacuation time of abalone (Britz *et al.*, 1996; Day and Cook, 1995; Foale and Day, 1992). Day and Cook, (1995) and Foale and Day, (1992) have shown that feeding frequency and diet quality influence the rate at which abalone digest algae. Less digestible algal species remain in the gut for more than 48 hours whereas preferred species are digested within 24 hours (Day and Cook, 1995; Foale and Day, 1992). Tahil and Juinio-Menez, (1999) showed a distinct nocturnal feeding periodicity in *Haliotis asinina* when fed algae, with the abalone remaining cryptic during daylight. They also found that defecation occurred during the feeding time in proportion to the amount of food consumed and with an increasing amount of feed, up to an equivalent of 35% of body weight, consumption increases. Studies on feeding behaviour of cultured abalone indicate that 60-80% of abalone emerge and feed every night on both natural and formulated feeds and that satiation is reached after 6 hours of darkness (Knauer *et al.*, 1995; Uki, 1981). On preferred feeds abalone reach satiation after two to four hours (Knauer *et al.*, 1995).

1.3.1. Natural Diet.

Red algae, of the Division Rhodophycophyta, are the natural diet of *Haliotis laevegata*. The food reserve of the red algae is a Floridean starch essentially similar to the branched type, or amylopectin, of higher plants (Meeuse, 1962). Other reserves recorded in the red algae include sugars and glycosides such as trehalose, floridoside, isofloridoside, maltose, and sucrose (Craigie, 1974). The cell wall components consist of xylans, cellulose and mannons (Bold and Wynne, 2000), with an outer component consisting of mucilage or slime. An outermost cuticle is primarily protein (Hanic and Craigie, 1969). The amorphous matrix of the wall is usually a sulphated galactan (Mackie and Preston, 1974), of which agar, porphyran, furcelleran and carrageen are examples. The lipid content is less than 2% (Britz *et al.*, 1996).

The red algal structure suggests large amounts of glucose would be available to the abalone if they have the digestive capacity to break down the complex sugars and cell wall components to simple molecules. The time taken for digestion and uptake is therefore important and this, as well as the relevant components of the gastrointestinal tract, may influence feeding behaviour and digestion.

1.3.2. Growth Rate

Comparison of growth performance of abalone on various diets is difficult because different species of abalone differ in their capacity for growth. Trial or experimental conditions such as the stocking density, water temperature, the initial size of the animals and the duration of the trial can influence growth. If a trial is short (<30 days) the growth data generated may not be as reliable as for a longer trial because the previous diet of the abalone has been found to influence the growth response when on the test diet (Viana *et al.*, 1996; Day and Fleming, 1992). The nutritional balance of a diet can only be assessed during trials of sufficient length to observe any effects of nutrient limitation. If a diet has a limiting nutrient, the abalone will use its own stores and once these are exhausted the growth rate of the animal may decrease or cease all together (Day and Fleming, 1992).

Because farmers use lengths to calculate time until harvest, it is common for growth to be presented as length increments of the shell during a certain period. Weight gain would be a better measure of the nutritional state of the animal as it will reflect the bulkiness of the foot and hence product quality. Dietary influences may also allow more shell deposition than deposition in the foot making shell length an unreliable indicator. However, for the following data, shell length is used for comparison because this is the most quoted growth indicator. In South Africa researchers have shown growth rates for *Haliotis midae* of 1.5-2mm/month (50-70μm/day) on diets of kelp (*Ecklonia maxima*) and 2.37mm/month (78μm/day) for an artificial diet of fishmeal and *Spirulina* sp. as protein sources (Britz *et al.*, 1994). Growth rates for abalone of size 7-20mm on artificial diets in different countries were: Japan 160μm/day, China 103-211μm/day, Australia 90μm/day and Mexico 120μm/day (Fleming *et al.*, 1996). In larger animals (25-50mm) growth rates range from 50-140μm/day and averaging 85μm/day in diets from the countries mentioned above (Fleming *et al.*, 1996). Growth rates on Australian farms vary, depending on the production systems, feed source and water temperature however the farmers require average growth rates of between 67-100μm/day to ensure abalone are at marketable length of 70-80mm in three years (Fleming *et al.*, 1999).

1.3.3. Protein Requirements.

Early work by Japanese researchers who fed graded levels of casein and fishmeal to *Haliotis discus hannai* found growth rate was positively correlated to levels of between 5 and 46% dietary

protein (Uki and Watanabe, 1992). Using feed conversion efficiencies, a measure of the amount of weight gained (g) by the animal per gram of feed intake, in this study it was found that protein at 28% inclusion was the suggested optimal protein level for the abalone. A control diet of the preferred algae for *Haliotis discus hannai* had a crude protein content of 17.5 %. The differences seen with the increase in protein and subsequent increase in growth rate, may not have been due to the protein level increases per se but rather to the change in protein to energy ratio. As the researchers increased the protein in these diets they correspondingly decreased energy producing dextrin levels. In a study using fishmeal as the dietary protein source Britz (1996) used inclusion levels from 27-47% and found a similar result to Uki and Watanabe (1992) in that as protein increased so did the specific growth rate. Cellulose was increased in the diets as the fishmeal was decreased and was assumed to be a non-nutritive filler. However, the increased cellulose may have confounded the results as Uki and Wantabi (1994) found that increasing cellulose from 0-20% decreased shell growth and weight gain. Therefore, the diets with the low protein level may have shown different results if an inert filler had been used instead of cellulose. Taylor (1992) concluded that a diet of 30% protein (casein) was necessary to produce maximal growth in *Haliotis kamtschatkana*. All the above trials were limited to the use of a single source of protein and it is unlikely that fishmeal or casein will provide all the essential amino acid requirements of the abalone for optimal growth.

The concept of an "ideal protein" source that contains a balance of amino acids has been advocated as a method to estimate the dietary essential amino acid requirements of animals in general. The basis of this concept is that there is a direct relationship between the amino acid profile of the soft tissue and the dietary amino acid requirements of the animal (King *et al.*, 1996). In conformity with this principal, in abalone, supplementation of amino acids in manufactured diets have been trialed in attempts to improve the protein quality by matching the amount of amino acid in the diet with those in the foot (Mai *et al.*, 1995a). Lysine (Hanna, 1992), DL-methionine (Gorfine, 1991), arginine and threonine (Knauer *et al.*, 1993; Vanderpeer *et al.*, 1998) have been trialed in abalone. In some commercial diets, amino acid supplementation is used, but the reasons for inclusion have not always been on sound nutritional knowledge (Fleming *et al.*, 1996). Mai *et al.*, (1995a) used the soft tissue amino acid profile of juvenile *Haliotis tuberculata* and *Haliotis discus hannai* to adjust their experimental diets for the abalone for which optimal growth was reported to occur between 25.2-36.6% protein for *Haliotis tuberculata* and 24-34.5% for *Haliotis discus hannai*. In this study the level of dextrin with

increasing protein was altered and hence the protein to energy ratio was changed. One parameter that was not determined in all the above studies was the digestibility of the individual protein source. The trials used crude protein and energy levels as the basis of the diets rather than digestible protein and digestible energy levels and as such the "ideal" levels reported may in fact be much lower. The wide range of ideal protein levels seen in the various studies may be a reflection of the capacity of the different abalone species to digest and assimilate the proteins.

1.3.4. Energy Requirements.

Abalone are poikilotherms and hence do not expend energy to maintain a body temperature differing from the surrounding water. They also spend little energy in foraging for food. They excrete waste nitrogen as ammonia for which little energy is required (Ruppert and Barnes, 1994). Energy losses in abalone are via faeces, ammonia, mucus production and as heat. In studies on *Haliotis midae* (Barkai and Griffiths, 1988) and *Haliotis tuberculata* (Peck *et al.*, 1987) energy losses from faeces were 63% and 20.7% of the energy intake for each species respectively and ammonia excretion was 1% for both species. Mucus production accounted for 23.3-29.1% of energy intake in *Haliotis tuberculata* (Peck *et al.*, 1987). Heat generated by *Haliotis tuberculata* from respiration ranged between 21.6-31.1% of the energy intake for a range of abalone size (Peck *et al.*, 1987) and with *Haliotis midae* 32% (Barkai and Griffiths, 1988).

Abalone consume a natural diet consisting of 40-50% carbohydrate; consequently carbohydrates are used as the primary source of energy. The metabolic rate of abalone is low so therefore the energy needs are low. Yamasaki, (1991) calculated the daily intake of gross energy in *Haliotis discus* to be between 0.3kJ-0.7kJ depending on temperature. Fleming (1991) found a maintenance requirement of 0.2-0.3kJ per day in *Haliotis rubra* and on preferred algae the daily intake of digestible energy was 1.2kJ (2kJ gross energy). Any excess energy in the abalone's diet is converted to glycogen in the foot (Webber, 1970). Glycogen levels have been reported as high as 41-48% in the foot of *Haliotis midae* (Knauer *et al.*, 1994). In starved *Haliotis kamtschatkana* glycogen reserves became depleted in the digestive gland and the foot muscle after 6 days and 27 days respectively however in gonads no change was shown (Carefoot *et al.*, 1993). Glycogen reserves are considered to enhance meat flavour and are an important consideration in diets used to attain marketable quality of the flesh (Fleming *et al.*, 1996; Bremner, 1994; Olaechea *et al.*, 1993).

Abalone meat is generally low in lipid, which is similar to their natural diet, with most lipids present in the cellular membranes as polar lipid (De Koning, 1966). The natural diet of Australian greenlip abalone is red algae which has the main C_{20} polyunsaturated fatty acids (PUFAs) 20:4(n-6) and 20:5(n-3) (Johns *et al.*, 1979). Many marine animals have only a limited capacity to chain-elongate and desaturate (n-3) C_{18} PUFA to corresponding long chain C_{20} and C_{22} PUFA (Dunstan *et al.*, 2001; Kanazawa *et al.*, 1979). Thus these animals have a specific dietary requirement for 20:5(n-3) and 22:6(n-3), the main PUFA in their tissues. In early work performed on *Haliotis discus hannai* a link was shown between diet lipid quantity and quality, in particular these C_{20} and C_{22} PUFA, to growth rates (Uki *et al.*, 1986). There is a requirement for n-3 and n-6 polyunsaturated fatty acids in abalone in particular 20:5(n-3) however unlike other marine animals, not 22:6(n-3) (Dunstan *et al.*, 2001; Dunstan *et al.*, 1996; Uki and Watanabe, 1992). The preferred food of newly settled abalone is diatoms that are high in 20:5(n-3) but low in 22:6(n-3) (Dunstan *et al.*, 1994a). Researches have found abalone only need low inclusion levels of oil. Wee *et al.*, (1992) report efficient lipid digestibility at 3.4% oil inclusion in *Haliotis laevegata*, while Uki *et al.*, (1986) found 5% lipid digestibility in *Haliotis discus hannai* and Van Barneveld *et al.*, (1998) and Dunstan *et al.*, (2001) 3% in *Haliotis laevegata*. Mai *et al.*, (1995b) found abalone fed 3% dietary lipid had a higher growth rate and also a higher soft body protein content in comparison to abalone fed high lipid levels. High levels of lipids (lipid level >6%) have been shown to decrease the digestion of dietary nitrogen, amino acids and energy in *Haliotis laevegata* (Van Barneveld *et al.*, 1998). As mentioned before abalone when feed-restricted are able to use glycogen as an energy source. This indicates that abalone may not use lipids for energy. Lipid uses are in maintaining normal cell functions, as phospholipid components of membranes, and in reproduction as precursors of prostaglandins (Sargent *et al.*, 1989).

1.3.5. Mineral Requirements.

Current mineral and vitamin levels are based on the requirements of the fin fishes, carp and rainbow trout (Uki and Watanabe, 1992) and are added to diets at approximately 3-4% (minerals) and 1.5-2% (vitamins) weight inclusion (Day and Fleming, 1992). An optimal level was found for mineral inclusion in the diet to be 8% in abalone based on an increase in growth with increasing mineral content up to 8% (Uki and Watanabe, 1992). The stability of the artificial feed pellet in water was improved with a 4% mineral inclusion and this compromise is generally used (Uki and Watanabe, 1992). A number of aquatic snail species absorb a large proportion of their extensive calcium requirements directly from the aquatic medium (Thomas and Lough,

1974) and it may be expected that abalone do the same. Phosphorous, like nitrogen, is a potentially limiting mineral in marine ecosystems (Smith and Atkinson, 1984) and an adequate supply in artificial diets is important. Coote *et al.*, (1996) reported an 8% increase in growth (length) with a 1% inclusion of phosphorous added to an artificial diet, however, supplementing calcium had no effect. It is important to keep the levels of phosphorous as low as possible to ensure that the water quality in the farm system is not compromised and that water discharged from farms does not contain excess levels of this nutrient. In an evaluation of mineral and vitamin requirements on *Haliotis laevegata*, Boarder and Maguire (1998) found that by increasing dietary vitamin mix 2 times (total 4% weight inclusion) above that used by Uki and Watanabe, (1992) improved growth, however they found when minerals were similarly increased there was a depression in growth unless vitamin inclusion levels were also elevated. Given that abalone have a different diet, physiology and behaviour to finfish there is an opportunity to refine the vitamin and mineral inclusion in artificial diets.

1.4. Diet Development

Ogino and Ohta, (1963) are often quoted as the first to investigate artificial diets in abalone. Their diet compositions were largely based on finfish diets with fishmeal being the protein source. Fishmeal is used extensively in many aquaculture diets as not only does it supply protein, it is palatable to many cultured animals and is a good source of energy and essential fatty acids (n-3 PUFA) and many essential minerals (Lovell, 1992). Soybean meal is considered to have the potential to replace fishmeal in aquaculture feeds because it has an amino acid profile similar to fishmeal and its protein is highly digestible (Evans, 1992). Casein has been trialed in diets and found to be a high-value protein source as it promotes growth and is highly digestible, however it is generally too expensive to be considered by feed manufactures (Uki and Watanabe, 1992). In addition fishmeal has been found to be generally superior to casein (Britz *et al.*, 1996; Viana *et al.*, 1993). A review of 12 diets by Fleming *et al.*, (1996) showed that most used either fishmeal, defatted soybean meal, casein or combinations of these as their protein source. Depending on the country of origin, the artificial diets also reflect the protein source. Casein use in New Zealand is due to access to milk by-products, which are low-cost in that country (Fleming *et al.*, 1996). In Australia the main protein sources used are soyflour and fishmeal. There is little locally-produced soy and fishmeal in Australia, almost all being imported at high cost (Vanderpeer *et al.*, 1999).

Because protein is essential for soft tissue growth and is the most expensive component in an artificial diet, researchers have concentrated their efforts on the protein component. The major factors affecting protein utilization are its digestibility, the balance of its amino acids and their availability, the amount of protein supplied and the amount of energy supplied. Energy sources come from low cost cereal sources such as wheat, cornflour, maize and rice starch. Starch is also used as a binder in commercial diets. Lipids supplied in artificial diets are fish oil, a vegetable oil or a combination of both.

One of the main problems encountered in abalone feeds is the necessity for the food particles of the diet to remain bound together in water for at least 2 days. The need to hold feeds together for extended periods are mainly because abalone are slow feeders. An additional requirement is that the feed needs to be of a consistency, which does not discourage feeding. Experiments by Uki *et al.*, (1985) using sodium alginate, which is extensively used as a binding agent in aquaculture feeds, reduced feed intake when content was increased from 30 to 50%. Similarly, Gorfine, (1991) found increases in agar from 2-8% decreased intake.

1.5. Australian Nutritional Research.

There are specific steps taken by commercial nutritionists in formulating animal diets to achieve maximum efficiency. These are to define the nutritional requirements of the animal, to access the nutritive value of individual ingredients and to formulate least cost diets based on the information gained in the previous steps. Van Barneveld, (1999) suggests using a stepwise approach in the development of aquaculture diets based on principles used in terrestrial nutrition research. These steps are 1: Develop a base diet focussing on form, acceptance, palatability, water stability, and shelf life and feeding strategies which minimize wastage, leaching and maximizing intake. 2. Develop an understanding of fundamental nutrition research principles. 3. Define nutritional value by nutrient digestibility and availability studies. 4. Define nutrient requirements by assessing the response to protein and energy intake, followed by amino acid, lipid, vitamin and mineral requirements. 5. Improve existing knowledge by applying current terrestrial nutrition techniques.

1.5.1. Base Diet.

In Australia a base diet has been formulated under the program initiated by the Fish Research and Development Council (FRDC) and abalone growers. This FRDC diet uses soyflour as its protein

source. Additives of algal species (*Graciliria*) at levels of 10% dry weight to the FRDC diet improved growth in both *Haliotis laevegata* and *Haliotis rubra* (Dunstan *et al.*, 1994b). A number of reasons may be attributed to the increase in growth. The acceptance and palatability of the diet may have improved, the algae may be more digestible and there may be nutrients in the algae at the required levels for abalone that were not adequately provided for in the FRDC diet. In 1995-6 farmers were experiencing high mortalities in some tanks that could not be explained by environmental factors. An analysis of body tissue showed cultured abalone had a vitamin A content of 7μmol/kg, much lower than wild-caught animals with 32μmole/kg (Fleming, 1996). Supplementing the FRDC diet with a micro alga containing β-carotene, which is a good source of vitamin A, improved growth by 15%, although Fleming (1996) suggests that other nutrients in the micro alga may have contributed to the improvement in nutritional value.

1.5.2. Diet Trials With Variable Nutritional Inclusion levels.

A trial of several artificial diets (protein sources casein, soybean meal, lupin meal) by comparison to the FRDC diet and a commercial diet in Tasmania on *Haliotis rubra*, showed the artificial diets using casein to be inferior to a diet of soybean meal and fishmeal as the protein source equivalent to the FRDC diet (Sharkie, 1997). This trial is an example of not having or using an understanding of fundamental research principles as described by van Barneveld (1999). The diet formulae do not appear to have a nutritional basis for the various inclusion levels and there are no reasons given for the formulation, which appear to be based on trial and error. There are many combinations of ingredients such that direct comparisons or specific effects of a single ingredient cannot be made. This trial appears to have failed to isolate the variables involved.

1.5.3. Nutrient Digestibility Studies.

The digestibility of a nutrient (protein, amino acids, energy source) is most accurately defined as that proportion not excreted in the faeces and is therefore assumed to be absorbed by the animal. These values provide apparent digestibility and not true digestibility as no correction is made for loss of endogenous nitrogen (McDonald *et al.*, 1999). In digestibility studies on abalone the collection of all faeces is difficult as the faeces produced are not always discrete pellets and can disintegrate in water. Under these conditions soluble nutrients can leach from the faeces and feed, causing problems in determining digestibility (Fleming *et al.*, 1996). The use of chromic oxide as an inert marker allows estimation of digestibility without the need to collect all the faeces, however there are a number of assumptions addressed. These assumptions are that the marker is

not absorbed into the gastrointestinal tract, it remains bound to the feed throughout passage in the gut and it does not effect the digestibility of the test feed (Fleming *et al.*, 1996). Digestibility trials must be conducted under strict temperature and light regimes because these factors have been shown to affect digestibility (Dixon, 1992).

As part of the FRDC-funded nutrition program on Australian abalone, investigations on the nutritive value of a range of local, readily available grains and legumes are being performed. Fleming *et al.*, (1998) performed digestibility studies on barley, semolina, lupin, casein and fishmeal, singly and in combination, with the aim of testing the assumption that digestibility of a single ingredient is additive when the ingredients are mixed in formulated diets. This group found that the digestibility coefficients of the ingredients were additive. They also found that no interactions occurred due to the non-starch polysaccharides present in barley and lupins in the mixed diets. The single-protein-source diets in this study were originally designed to be isonitrogenous however difficulties in collecting faecal samples caused the researchers to increase the protein levels to minimum inclusion levels of 70%. High inclusion levels of fish and shrimp meal (Brown *et al.*, 1989; Law *et al.*, 1990; Reigh *et al.*, 1990; Shiau *et al.*, 1992) or high starch plant meals (Allan *et al.*, 1998; Ashmore *et al.*, 1985; Davis and Arnold, 1993; Pfeffer *et al.*, 1995) have been shown to adversely effect the digestibility's of reference feeds, which may be the case in this study. It is surprising that the impact of dietary fibre, specifically the non-starch polysaccharides, in barley (Dahle *et al.*, 1992; Klopfenstein, 1988) and lupin (Evans *et al.*, 1993; Saini, 1989; Watkins *et al.*, 1988) showed no effect. By comparison it is well documented that the efficiency of protein and energy components of diets is reduced with non-starch polysaccharides in chicken (Annison, 1991; Choct and Annison, 1992) rats (Hansen *et al.*, 1991; Zhao *et al.*, 1996) pigs (Bach Knudsen *et al.*, 1993; Van Barneveld *et al.*, 1995) and prawns (Sarac *et al.*, 1993). The most notable effect of non-starch polysaccharides in diets is an increase in digesta viscosity and this was considered the main mechanism by which non-starch polysaccharides influence productivity (Salih *et al.*, 1990; Smits and Annison, 1996). Both of the above effects may have been seen in the study of Fleming *et al.* (1998), given the high inclusion levels used, and as such their results would need to be considered with caution.

Vanderpeer *et al.*, (1998) formulated five diets which differed in their level of inclusion and combinations of 4 ingredients, semolina, soyflour, casein and fishmeal to specified protein and energy levels to prove the reliability of individual digestibility coefficients already known for

these ingredients. They found that similar growth rates were produced by the five diets and demonstrated that the individual ingredient apparent amino acid and energy digestibility coefficients can be used confidently in formulating abalone diets to specific protein and energy levels. In this study, because they used specified protein and energy levels, the calculated and experimental results should be similar unless other factors affected their results such as ingredient interaction.

1.5.4. Protein to Energy Ratios.

Coote (unpublished thesis, 1997) conducted an experiment investigating the effects of different digestible protein to digestible energy ratios on the growth and body composition of greenlip abalone. Ten levels of dietary protein were trialed at two energy levels (Van Barneveld *et al.*, 1998). Abalone fed the higher-energy diets grew significantly slower than those fed the lower energy diets at all protein levels. The higher-energy diets contained approximately 50g/kg more oil than the lower energy diets. A subsequent experiment by Van Barneveld *et al.*, (1998) showed that these high levels of oil decreased the digestion of amino acids and energy. This experiment should be performed again with recommended oil inclusion levels of Van Barneveld *et al.*, (1998) to establish a true indication of the digestible protein to energy ratios.

1.5.6. Current Nutritional Investigations.

In keeping with overall goals of reducing feed costs to the industry, interest has increased in the use of locally grown legumes (faba beans, lupins, peas and vetch), as alternatives to soyflour and fishmeal. Costs per tonne (1999) are: soyflour $A950-$1000, fishmeal $A1600-$1800, faba beans $A215, lupins $A160, peas $A220, and vetch $A310, the legumes being much cheaper (Vanderpeer *et al.*, 1999). Vanderpeer *et al.*, (1999) have completed preliminary digestibility studies on the cheaper alternative protein sources of faba beans, lupins, pea and vetch which indicated that lupins may be a suitable feedstuff for use in artificial diets for abalone.

Legumes have been used in stock feeds and have been the subject of many studies. Anti-nutrient factors associated with the legumes such as trypsin inhibitors, protease inhibitors, alkaloids, lectins and tannins have been the main problem in using these products. Phytic acid associated with plant feedstuffs has been linked with phosphorous deficiency due to phosphorous being indigestible in this form (Reddy *et al.*, 1982). Raw legumes and those treated by autoclaving, dry heat-treatment (cooking) and enzyme supplementation have been tested in both terrestrial and

aquatic species (Irish and Balnave, 1993); (Brenes *et al.*, 1993; Irish and Balnave, 1993; Longstaff and McNab, 1987; Pfeffer *et al.*, 1995; Ressler *et al.*, 1968; Rubio *et al.*, 1990; Rumsey *et al.*, 1993). The effects/benefits can be species specific (Huisman and van der Poel, 1988).

There are a number of enzymes available for use in animal feeds that could be useful in abalone artificial diets. Proteases, cellulases, lipases, pectinases, β-glucanases and phytates all may assist digestion by abalone. The work of Coote *et al.*, (1996) on phosphorous has led to trials of phytate in a legume based trial by Vanderpeer *et al.*, (1999) to confirm Coote's results. Enzyme supplementation may be most useful in high fibre diets in which the fibre (cellulose) is used for binding purposes.

Under the stepwise approach suggested by Van Barneveld the research is at step 3, i.e. define nutritional value, with some work completed in step 4, i.e. define nutrient requirements. However this work has areas that should be reassessed in the light of later studies. The success of nutritional work has led to a world-class artificial diet for abalone in Australia, even though there is still a number of areas that need addressing and areas that could benefit from further studies.

1.5.7. Growth Trials.

There are a few limitations to digestibility studies. Once a digestibility coefficient of an ingredient is obtained a diet can be formulated by applying these values to specific digestible protein or energy requirements found for the animal. Because it is unlikely that a single protein source may supply all the amino acids required by an animal at the correct levels for maximum growth, several protein sources are generally used in diets and these are assumed to be additive (Cho and Kaushik, 1990). Wilson and Poe, (1985) found this additive effect in experiments with channel catfish using feed ingredients of corn, Menhaden fishmeal, soybean meal and wheat. As mentioned previously, Vanderpeer *et al.*, (1998) also found this additive effect with *Haliotis laevegata* using semolina, casein, Peruvian fishmeal and defatted soyflour. In contrast, Lupatsh *et al.*, (1997) formulated five diets with equal digestible protein to energy ratios, using eight ingredients, for feed trials with gilthead sea bream, results showed that the digestibility of carbohydrates varied as much as 90% between diets. The reasons given were the small inclusion levels of carbohydrates and interactions of them in the diets affecting digestibility. Diets with protein-rich animal meals and carbohydrate-rich plant meals appear to have the greatest effect on feed ingredient digestibility (Lee and Lawrence, 1997). As mentioned previously, the percent

inclusion of protein and energy sources as well as non-starch polysaccharides can all effect digestibility and hence growth rate. Micronutrient interactions including vitamin/vitamin, vitamin/mineral and mineral/mineral can occur (Hilton, 1989). Micronutrients can interact with macronutrients for example vitamin E with polyunsaturated fatty acids, thiamin/carbohydrate, zinc/calcium/phosphate and pyridoxine/protein (Hilton, 1989). Growth experiments are the only way to establish if there are interactions and to see if the digestibility coefficients are indeed additive. Therefore, nutrient digestibility coefficients can only give an indication of the contribution of available nutrients and energy from each ingredient, not the productivity of the ingredients in the whole diet.

Abalone have a long grow out period (2.5-3 years) in comparison to terrestrial farmed animals, chickens (6-8 weeks), pigs (12 weeks), cattle (8-10 months) and therefore a diet for abalone should not only provide for suitable growth but should also maintain the animal in optimum health. Digestibility studies and growth trials have been successful in aiding the formulation of diets that have provided the production requirements of many animal species. While a diet may produce good growth, the impact on the digestive tract especially over a long period, cannot be seen in growth studies. To gain the most effective assessment of production requirements of the animal, knowledge of the digestive capacity and the effects that diet can have on the digestive tract needs to be studied. Other than enzyme profiles, mainly in the gut prior to the intestine, this area has not been studied in abalone and very little is known of the digestive capacity of the intestine, and uptake mechanisms for food.

1.6. The Structure and Function of the Gastrointestinal Tract of Abalone.

There have been general anatomical accounts of Haliotids, mainly the European *Haliotis tuberculata* during the 19th century and early 20th century with the most complete by Crofts, (1929). Bevelander, (1988), in his book, shows gross and fine structure of the abalone while Campbell, (1965) describes both structure and function. The following structural and functional characteristics are mainly taken from these sources.

Haliotis species are both coarse and fine particle feeders, which suggests that the digestive system, may function in handling both grades of material. The digestive tract of *Haliotis* has a buccal region bearing the radula followed by the oesophagus, crop, stomach, caecum, hepatopancreas (previously known as the digestive diverticulum) and intestine.

The buccal region has a prominent radula and chitinous jaws, which provide a firm surface against which the radula works. These jaws help the mouth retain pieces of algae obtained from scraping and these particles are conveyed to the buccal cavity. Mucous and secretions from salivary glands located near the radula are mixed with food particles and facilitate the movement of these particles into the oesophagus (Campbell, 1965; McLean, 1970a).

The oesophagus leads from the buccal cavity with the posterior oesophagus widening into a roomy ventral chamber known as the crop (Bevelander, 1988) or first stomach (Crofts, 1929). Algae are partially digested in the crop with some algae degraded to an unrecognisable state within six hours of ingestion (Foale and Day, 1992). Posteriorly the crop constricts with a narrow opening, which then continues as the stomach. The crop and stomach show similar peristaltic contractions or movements with the contractions strong and uniform. The stomach has less than half the capacity of the crop (Crofts, 1929). The stomach proper has a spiral caecum, which receives the secretions of the hepatopancreas. The movements of cilia and the strong peristaltic contractions of the stomach move food into the caecum in which sorting of material occurs (Campbell, 1965). This material then emerges as threads that form the rotating mass of coarse particles and faecal material in the stomach. The contractions of the stomach wall force fine particles into the hepatopancreas that contains enzymes for the metabolism of lipids, carbohydrates and proteins (Bevelander, 1988). A narrow opening combined with strong ciliary movements keeps large particles from entering the intestine allowing only fine particles to enter. The stomach then leads into the style sac, which extends to the start of the small intestine.

The intestine has been described as five sections largely based on where contortions or distinctive changes in villus structure are found. The intestine extends around the right side of the foot muscle reverses sharply beyond the anterior most point of the muscle, then passes back to a point below the ventricle. The intestine then reverses to pass through the ventricle and terminates at the anus within the mantle cavity between the gills (Campbell, 1965). The *Haliotis* intestine is long, and provides ample surface for digestion and absorption according to Bevelander (1988), however, Campbell (1965) sees its function as an area for organizing faecal rods. The intestine has fewer cilia than the oesophagus suggesting a slower food passage allowing ample time for phagocytes seen in this area to absorb food (Harris *et al.*, 1998c). Region 5 of the small intestine, often referred to as the rectum, has the greatest density of mucus cells of any part of the intestine. The secretions from the mucous cells cover the compacted faecal pellets. Cilia in this region

direct the faecal pellets to the anus (Campbell, 1965). Compaction of the faecal pellets aids in preventing the disintegration of the pellets in the mantle cavity of the animal (Fretter and Graham, 1976).

The above accounts conflict as to the function of the intestine in abalone. To understand nutrient digestion and uptake in abalone this area needs to be resolved to define what capacity the abalone has in digesting food and subsequent uptake. Understanding the developmental changes and general morphology of the abalone intestine may provide knowledge that may also aid diet development. As there has been little or no work conducted on abalone in respect to intestinal function the following accounts rely predominantly on the extensive knowledge gained in mammalian, fish and poultry studies.

1.7. Intestinal Development.

In mammals and fish, the development of the gastrointestinal tract is generally along three axes. Some of the changes occur with the age of the animal. Observed changes that have been noted are on the horizontal axis, the length of the intestine, as well as the vertical axis, from the crypt to the villus tip. These changes regulate the efficiency of the gastrointestinal tract through modifications to the surface area available for digestion and absorption. Mucosal changes in the intestine can be profound. Overall these changes involve extensive proliferation of epithelial cells and cyto-differentiation, including changes in the expression of receptors, enzymes and transport systems (Kelly *et al.*, 1992). The interactions of endogenous and exogenous factors regulate these changes.

The growth of most body organs, including the gastrointestinal tract, occurs by cellular hypertrophy and/or hyperplasia (Morisset, 1993). All tissues made of parenchymal cells grow by cellular hypertrophy and/or hyperplasia. The intestinal tissue differs from many tissues in that it is renewable (Leblond, 1972). Such mucosal changes can be directly assessed by measurements of organ weight or indirectly through concentrations of nucleic acids and protein or the specific activities of local enzymes (Waterlow *et al.*, 1978).

1.7.1. Age related.

In mammals, intestinal mucosal growth can differ between neonates and adults such that nutrient uptake in neonates can occur along the entire crypt/villus axis while in adults uptake is at the villus apex (Smith, 1981). Also the enterocyte lifespan is longer in neonate pigs (Jarvis *et al.*,

1997) and the migration rate of cells along the crypt/villus axis is slow in neonate rats (Koldovsky *et al.*, 1966). In neonate mice, mitosis may occur along the entire crypt/villus axis (Beaulieu and Calvert, 1987) whereas in the adult it occurs only in the crypt.

The response of the intestine to feeding or fasting may vary with age. Enzyme-specific activity of sucrase, maltase and lactase is lower in the proximal intestine in adults rats in comparison to neonate rats (Holt *et al.*, 1985). During fasting the internal mass of the intestine was reduced more severely in adults than in young rats and with re-feeding the activities of sucrase and maltase responded more quickly in adults (Holt and Kotler, 1987; Reville *et al.*, 1991). Age may also be a factor in the profiles of enzymes in abalone, for example Spaulding and Morse, (1991) found two arylsulfatases in competent veliger larvae and four in the hepatopancreas of adult abalone. They suggested that this is due to dietary changes at metamorphosis where abalone move from the planktonic (suspended in water) to the benthic habitat and the concomitant switch in energy acquisition from lecithotroph (non-feeding larva using yolk) to herbivore. Uki and Watanabe, (1992) fed heat-treated fishmeal to small (13mm) and larger (52mm) *Haliotis discus hannai*. The results showed the smaller abalone digested this diet more efficiently than the larger abalone and proposed that this may be a specialization of decreased protease activity with age due to the smaller abalone requiring more protein for growth.

Transport rates can also be age dependent. Amino acid uptake can vary in that a decline in lysine, methionine and glucose uptake in pig occurred postnatally (Smith, 1988). In chicks glucose uptake increased with age up to 14 days while oleic acid uptake remained unchanged (Shehata *et al.*, 1984).
Manahan and Jaeckle, (1992), in studies on abalone larvae that lack a digestive system, showed that the larvae simply absorbed dissolved amino acids and carbohydrates from the surrounding water. No reported evidence on mechanisms of nutrient uptake in juvenile or adult abalone is in the literature.

1.7.2. Horizontal axis.

There are physical differences along the horizontal axis (length) in the abalone intestine in that three distinct sections can be seen. The first one third (approx) has a larger circumference with 11-13 distinct ridges and a typhlosole; the next section accounts for 60% of the length of the intestine and is characterized by a typhlosole and a smaller circumference. The final section is

the area where faecal pellets are formed. No separate studies have been done on the first and second section of the intestine to see if there are differences along this horizontal axis.

In mammals structural and functional changes may occur at different rates for the regions of the intestine and they are important in the response to dietary or disease factors as well as the digestive and absorptive capacity (Harrison and Webster, 1971). In pigs the proximal intestine has longer villi than those in the distal regions (Hall *et al.*, 1983) which is also be seen in the chicken (Moon and Skartvedt, 1975). Digestive enzymes, such as sucrase and alkaline phosphatase, generally have greater specific activity in the proximal intestine rather than in the distal regions (Amat *et al.*, 1992; Holt and Kotler, 1987). Other enzymes such as leucine aminopeptidase in chickens show similar activity over the entire small intestine (Amat *et al.*, 1992) whereas carboxypeptidase P in rats shows higher activity in the middle section (Erickson *et al.*, 1989). Transport rates of amino acids can vary along the horizontal axis as shown in the rabbit where lysine, leucine, and β-alanine showed a lower rate in the jejunum and an increased rate in the ilium (Munck and Munck, 1992).

1.7.3. Vertical axis.

The intestinal mucosa is renewable in that enterocytes originating in the crypts migrate towards the apex of the villus where they are extruded into the lumen. Approximately 30-50% of the endogenous protein entering the small intestine comes from the replacement of epithelial cells lining the small intestine (Smith, 1990). The life span of these cells can vary between species, being 2 days in the rat (Syme and Smith, 1982) and 2-3 days in the chicken (Spielvogel *et al.*, 1972). This high rate of cell turnover is presumably needed in order to maintain the barrier function of the epithelium and enable the intestine to adapt rapidly to changes in the local environment. The physical size of the crypts in mammals controls the mode of differentiation of the enterocytes as well as the rate of expression of digestive enzymes whereas the rate of migration influences their functional capacities (Morrill *et al.*, 1989). The protein content of enterocytes increases as cells migrate up the villus (Morrill *et al.*, 1989). Peak specific enzyme activity varies along this axis with sucrase and maltase observed near the crypt in rats and alkaline phosphatase at the upper region of the villus (Holt *et al.*, 1985). Nutrient transport systems generally function in the upper region of the villus (Debnam *et al.*, 1990; King *et al.*, 1981) as it is considered that the enterocytes complete microvillus development before they are able to absorb nutrients (Smith, 1990). Transport systems may be induced by nutrients, for

example, peptidase activity and amino acid transport activities have been shown to depend on the amount and/or type of protein present in the diet (Smith, 1990).

1.7.4. Effects of Diets on the Intestine.

Diets that are not well balanced, in that protein and energy is not optimal, ingredients contain non-starch polysaccharides and/or other anti nutrient factors, can change the pattern of intestinal development and function. Physical differences can be seen in the height and width of villus structure with dietary deficiencies or excesses. In rats on low protein diets the villus basal width increased, there was a thinning of the intestinal wall and there was an increase in production of crypt cells (Syme, 1982). Adaptation to low protein diets can occur by reduced mitotic rates and villus height or a combination of both (King *et al.*, 1983; Syme and Smith, 1982) with changes in villus structure always accompanied by changes in crypt cell proliferation rates (King *et al.*, 1983; Smith and Peacock, 1989). Should energy and protein supplies become limited there can be a loss of cellular albumin, which creates a situation where infections by enteropathogens may increase (Syme and Smith, 1982). In contrast to this, high energy diets in both pigs and chickens induce a rapid growth of the intestinal mucosa (Moore *et al.*, 1989; Yamauchi *et al.*, 1996).

The use of cereals and legumes in diets as protein sources have been shown to have deleterious effects that reduce the growth and health of an animal. Anti-nutrient factors associated with these sources have shown effects on the intestinal structure as well as interfering with enzyme function. The effects can be species-specific (Huisman and van der Poel, 1988) and depend on differences seen in chemical composition, the molecular sizes and solubility in water of the anti nutrient factor (Smits and Annison, 1996; Ward, 1996). The inclusion of pectin in diets reduced villus height, increased crypt depth and reduced enterocyte life span (Jacobs, 1983). Starch is the predominant carbohydrate used for energy in diets. Some of the carbohydrates are in the form of non-starch polysaccharides the most common being β-glucans, fructans and arabinoxylans (Classen and Bedford, 1991). Non-starch polysaccharides when digested yield short chain fatty acids that induce cellular proliferation and mucosal growth (Mathers and Kennard, 1993; Tulung *et al.*, 1987) and may do this through the regulation of insulin (MacDonald *et al.*, 1991). The most notable effect of non-starch polysaccharides in diets is an increase in digesta viscosity and this is considered the main mechanism by which non-starch polysaccharides influence growth and health (Salih *et al.*, 1990; Smits and Annison, 1996). Wheat bran caused shortening and atrophy of the microvilli in rat intestine and was linked to the presence of trypsin inhibitor

(Jacobs and Schneeman, 1981). Many plant protein sources contain protease inhibitors that reduce the activity of intestinal enzymes by forming chelates with the endogenous enzymes (Petterson and MacIntosh, 1994; Trevino *et al.*, 1992).

1.7.5. Anti-Nutrient Factors in Legumes.

Anti-nutrient factors associated with the legumes, such as trypsin inhibitors, protease inhibitors, and alkaloids, lectins and tannins are the main problem in using legumes in diets. Responses to anti-nutrient factors include a decrease in feeding efficiency, growth inhibition, goitrogenic responses, pancreatic enlargement and organ damage (Saini and Batterham, 1988).

A few legume seeds have demonstrated protease (trypsin) inhibitor activity (Petterson and MacIntosh, 1994). Growth-depressing effects are seen in certain legume seeds and have been traced to the presence of toxic factors, rather than a deficiency in amino acids (Petterson and MacIntosh, 1994). Tannins in faba beans and cowpeas, are the limiting anti-nutrient factors which complex with starch and proteins reducing digestion (Trevino *et al.*, 1992). High levels of manganese, the toxicity of which is characterized by depressed appetite and retarded growth, have also attributed to the low nutritive value of lupins (Cheeke and Kelly, 1989). Phytic acid, containing considerable phosphorous, associated with plant feedstuffs is linked with phosphorous deficiency due to it being indigestible in this form (Reddy *et al.*, 1982). Phytate also forms protein-phytic complexes that may reduce the availability of protein and minerals such as manganese, copper, molybdenum and zinc (National Research Council., 1999). Certain anti-nutrient factors are heat labile. Significant improvements in metabolisable energy, decreases in pancreas size, increased feed utilization and increased protein and starch digestibility have been found in poultry when fed heated legumes compared to those fed unheated legumes (Brenes *et al.*, 1993; Conan and Carre, 1989; Igbasan and Guenter, 1996; Marquardt and Ward, 1979).

Anti-nutrient factors can be reduced by various treatments. Trials, using treatments of raw, autoclaved, heat-treated (cooking) and enzyme supplementation on legumes, have been conducted in both terrestrial and aquatic species (Brenes *et al.*, 1993; Irish and Balnave, 1993; Longstaff and McNab, 1987; Pfeffer *et al.*, 1995; Ressler *et al.*, 1968; Rubio *et al.*, 1990; Rumsey *et al.*, 1993). The effects/benefits to animals can be species specific (Huisman and van der Poel, 1988).

1.8. Digestion in the Intestine.

Foods cannot be absorbed in their natural forms through the gastrointestinal mucosa, rendering them useless for nutritional purposes without the preliminary process of digestion. Digestion uses both physical events of chewing and emulsification as well as chemical breakage of covalent bonds by mineral acids and enzymes. Hydrolysis is the basic process of digestion. Specific hydrolysing enzymes for different carbohydrates, fats and proteins occur in the intestine and aid digestion. Later stages of digestion occur through enzymes located in the brush border of the intestinal epithelia.

Most of the identified mammalian intestinal enzymes are integral proteins bound to the epithelial membrane, however some are only partially anchored (Kenny and Turner, 1987). Intestinal enzymes are asymmetrically orientated with the catalytic site exposed at the surface of the cell (Kenny and Turner, 1987).

Most peptidases are metallo-enzymes linked to Zn^{2+} with more aminopeptidases found than carboxypeptidases (Kenny, 1986). Intestinal enzymes are responsible for the terminal digestion of substrates formed from pre intestinal digestion of food and include disaccharides and peptides. The secretion of enzymes appears to be at the crypt but expression is mainly on the villus (King *et al.*, 1983). Maximum activity of aminopeptidase and isomaltase in rat intestine was located at the crypt-villus junction (King *et al.*, 1983).

1.8.1. Enzymes in Abalone.

The majority of the studies on abalone digestive enzymes have used the hepatopancreas or a combination of the crop and hepatopancreas. Carbohydrate hydrolytic enzymes are present in many *Haliotis* species. Among those reported are alginic acid, cellulase, fucosidase, arylsulfatases, trehalase, amylase, laminarinase and carrageenanase, the presence of which can be related to the structural polysaccharide linkages commonly encountered in natural diets (Bennett *et al.*, 1971; Boyen *et al.*, 1990; Clark and Jowett, 1978; Knauer *et al.*, 1996; McLean, 1970b; Nakada and Sweeny, 1967). Bennett *et al* (1971) and Clark and Jowett (1978) have found maltase, sucrase and β-galactosidase in these regions.

Abalone, like many other organisms, cannot synthesis 10 of the 20 amino acids required to assemble proteins (Campbell, 1965). Studies on protein digestion in abalone are meagre. Groppe

and Morse, (1993) found chymotrypsin-like proteases in the distal intestine of *Haliotis rufescens* as well as trypsin activity. Serviere-Zaragosa *et al.*, (1997) found the highest proteolytic activity of trypsin and chymotrypsin to be in the intestine of *Haliotis fulgens*, with chymotrypsin 10 times higher than trypsin. They also found several protease activities in the hepatopancreas and stomach, but no description was given. Herandez-Santoyo *et al.*, (1998) found three serine protease-like enzymes in aqueous extracts from the hepatopancreas of *Haliotis fulgens*, one chymotrypsin-like, two trypsin-like as well as one carboxypeptidase.

Lipases have been little studied, but very low levels have been reported by Knauer *et al* (1996) using the total gastrointestinal tract rather than localized area studies. Britz *et al* (1996) showed very low activity of lipases and they found no link in lipase activity to food consumption. Lipase activity being low, and the fact that reserve energy in *Haliotis* tissue is stored in the form of glycogen, suggests that their ability to utilize lipid for energy appears to be limited (Britz *et al.*, 1996; Fleming *et al.*, 1996; Knauer *et al.*, 1996; Uki and Watanabe, 1992).

The wide variety of carbohydrases which abalone possess enables an animal to digest structural and reserve carbohydrates in their natural diet. This provides strong indications that carbohydrates from terrestrially grown sources may be utilized by abalone, provided that they are not complexed in ways which would make them insoluble or indigestible. The fact that protease activity is greater in the intestine than in other sections of the gastrointestinal tract, as shown in mammals, may lead us to hypothesize that final carbohydrate digestion could also be found here in abalone. Contrary to Campbell's (1985) view of the intestine, which is, 'an area to organize faecal rods', it may be a significant area for final digestion and uptake of nutrients which is the case in mammals. With this in mind it would also be reasonable to use techniques often used in mammals such as histological identification of areas of functional enzyme activity in the intestinal and to isolate apical membranes for identification of specific activity of enzymes and possible transport systems. The information gained from such studies would probably improve diet formulation as well as allow the comparison of mammalian studies on interactions between dietary ingredients as a basis for abalone studies.

1.8.2. Enzyme Diet and Species Interaction Effects.

Reports of the enzymatic capabilities of the southern hemisphere *Haliotis iris*, which prefers red algae, indicates that this species can hydrolyse a wide range of polysaccharides from red algae

but not some brown algal polysaccharides (Clark and Jowett, 1978). By contrast the Japanese abalone *Haliotis discus* (Yamaguchi *et al.*, 1989), *Haliotis discus hannai, Haliotis siebaldii* and *Haliotis japonica* (Nakagawa and Nagayama, 1988) all show a poor ability to degrade agar and carageenan, both red algal polysaccharides not common in their natural diet of brown algae. The Californian abalone *Haliotis cracherodii* and *Haliotis rufescens* can digest the cell walls of both red and brown algae (Fleming *et al.*, 1996).

These studies indicate that abalone contain many types of carbohydrases, which make them highly efficient at digesting the complex polysaccharides in algae. The enzyme profile may vary considerably between species with different natural dietary preferences. In any particular species, the range of novel feedstuffs may be limited by the carbohydrate components suited to the species.

1.8.3. Diet Related Enzyme Changes.

There is some evidence that abalone alter their enzyme profile to cope with changes in diet. In a study comparing a diet of diatoms and an artificial diet with protein sources of fishmeal (15%) and casein (5%), Knauer *et al* (1996) found that abalone fed the artificial diet had higher protease and lower α-amylase activities. Taylor (1994) demonstrated in *Haliotis kamtschatkana* enzymatic adaptation to varying levels of carbohydrates and lipids in artificial diets. When abalone were pre-fed either red or brown algae diets they showed digestion of each alga to be more efficient in abalone which had been pre-fed that particular alga compared with those that had not been pre-fed (Fleming *et al.*, 1996). This suggested that the enzyme composition or capacity altered over time resulting in greater digestive efficiency. In *Haliotis midae*, Erasmus (1996) found that abalone synthesise carboxymethylcellulase, laminarase, alginase, carrageenase, agarase and that in abalone fed different algae a positive correlation showed between the synthesis of enzymes and the diet. Abalone fed the algal species *Ecklonia* (which contains relatively high concentrations of alginate and laminarin) exhibited significantly higher alginase and laminarinase than abalone fed *Gracilaria* (which contain low levels of the above). Similarly, abalone fed *Gracilaria* (which contains relatively high proportion of carageenan and agar) produced significantly higher carrageenase and agarase activity.

1.8.4. Enzymes of Microbial origin.

Although evidence shows that abalone produce enzymes that cope with the digestion of algae efficiently, the significance of the role of gut bacteria is unknown. Erasmus *et al.*, (1997) found enteric bacteria in the crop, stomach and intestine of abalone, the common genera identified being *Vibrio, Alcaligenes, Flavobacteria, Pseudomonas* and *Aeromonas*. *Alcaligenes* occurred mainly in the crop while *Vibrio* species were predominating in the intestine. All bacteria showed the capability of using two or three of the substrates tested with carageenan and agarose hydrolysis in the intestine and alginate, carboxymethylcellulose and laminarin in the crop. Harris *et al.*, (1998d) found 44 isolates of bacteria within the gut of *Haliotis laevigata*. All were capable of degrading algal polysaccharides, starch, carboxymethylcellulose and agar, at pH similar to that of the different regions of the abalone gut. Harris *et al.* (1998d) therefore suggested that bacteria might play a role in abalone digestion but to what degree remains unclear. Both Erasmus *et al.* (1997) and Harris *et al.* (1998) found that there were no bacteria associated with the gut wall and they suggested that most of the bacterial digestive activity is restricted to the lumen.

The endogenous and exogenous bacterial sources of enzyme profiles of abalone indicate they have a considerable capacity to cope with their natural diets although these appear to be somewhat species specific. It may be necessary to summarize and measure enzyme type and capacity in individual species to match terrestrial diet sources with the different abalone. How and where abalone uptake the digested material from enzyme degradation as mentioned before is an important question to answer.

1.9. Nutrient Absorption in the Intestine.

If an organism is to live and grow it must obtain nutrients and other substances from the intestinal lumen and through a vectorial process pass it into a cell for processing or exchange to the vascular system (Hopfer *et al.*, 1973). Most substances pass through cell membranes by diffusion and active transport, but for most nutrients, the active transport mechanism predominates (Webb, 1990). At high substrate concentrations the contribution of simple diffusion to total transport is enhanced, depending on the permeability of the membrane to the nutrient (Stevens *et al.*, 1984; Webb, 1990). At high concentrations of glucose the quantity of glucose absorbed can be two to three times greater than saturated Na^+ co-transport through the enterocyte itself (Stevens *et al.*, 1984). Very large molecules enter the cell by a specialized process called endocytosis. The principal forms of endocytosis are pinocytosis (small molecules) and

phagocytosis (large molecules) where vesicles form in the enterocyte membrane entrapping molecules inside. The amount of endocytosis in adult mammals is small in comparison to total absorption by transmembrane transport protein (Smith, 1990). Transport of molecules can also be by 'solvent drag' through the cell junctions into the paracellular spaces. This transport usually occurs at high concentrations of a molecule in the lumen (Stevens *et al.*, 1984; Webb, 1990). Campbell (1965), Bevelander (1988), and Harris *et al.*, (1998) have reported phagocytes in the abalone gastrointestinal tract and Harris *et al.*, (1998) found fewer phagocytes in the stomach and intestine of starved abalone and saw this as a response to decreased food availability.

In abalone, a small number of researchers suggest uptake is by phagocytosis, however, the other mechanisms of nutrient uptake have not been studied (Campbell, 1965; Bevelander, (1988); Harris *et al.*, 1998). The possibility that intestinal transport mechanisms, seen in both terrestrial and marine animals, are also present in abalone should not be excluded.

1.10 Adaptation to Dietary Effects on the Intestine.

In species that have been more extensively studied, the gross effects of adverse dietary regimes have been partly attributed to changes in the pattern of intestinal development and function. These effects can be seen immediately or in the short and long-term adaptation to a diet, as previously discussed (sections 1.7.4, 1.7.5, 1.8.3, 1.9).

Immediate and short-term adaptations are seen with enzyme activity, transport and tissue changes. Changes to enzyme activity vary with both reductions and increases noted. These changes may be due to fasting (Reville *et al.*, 1991), refeeding or altered diets (Holt *et al.*, 1986; Morrill *et al.*, 1989), protein levels (Zambonino-Infante *et al.*, 1989) changes in diet (Hall and Byrne, 1989) and, lastly, effects of anti-nutrient factors which bind to digestive enzymes to reduce enzyme activities and digestion (Ikeda and Kusano, 1983; Petterson and MacIntosh, 1994). Also, the formation of complexes can cause nutrients to be unavailable: examples being phosphorous bound in phytic acid (Reddy *et al.*, 1982), protein-phytate complexes reducing several proteins and minerals (National Research Council., 1999), β-glucans and other non-starch polysaccharides may bind to dietary nutrients as well as reduce nutrient mobility, impairing digestion and absorption (Klopfenstein, 1988; Read, 1987) and tannins complexing with starch and protein reducing digestion (Trevino *et al.*, 1992). Tissue changes can be denudation of apical surfaces and shrinkage of villi due to fasting (Yamauchi *et al.*, 1996; Dauncey *et al.*, 1983) as

well as villus and crypt hypoplasia through reduction in cell proliferation and migration rates (Holt *et al.*, 1986). Several non-starch polysaccharides induce increases in intestinal weight through an increased rate of cell proliferation (Brunsgaard and Eggum, 1995; Johnson and Gee, 1986).

Macromolecule transport may be more pronounced on unnatural diets that do not provide the nutrient requirement of the animal (Segner *et al.*, 1987). The regulation of rates of amino acid transport can be enhanced by an increase in the concentration of amino acids in the lumen (Stevens *et al.*, 1984) and in some cases by substrate starvation (McGivan, 1996) as well as hormonal regulation (Chen *et al.*, 1987). Transport may also be effected by ion losses in the epithelium due to increased permeability through the cell membrane (Schwartz *et al.*, 1982).

Long-term adaptations to diets are dependent on the particular type of nutrient deficiency in the diet (eg protein or energy). The morphological response to severe protein deficiency is an increased production of crypt cells, reduced mitotic rate and villus height and a thinning of the intestinal wall (King *et al.*, 1983; Syme, 1982; Syme and Smith, 1982). When energy supply becomes limiting a higher risk from infection can occur (Dosseter and Shittle, 1975; Syme and Smith, 1982). High-energy diets induce a rapid growth of the intestinal mucosa (Moore *et al.*, 1989; Yamauchi *et al.*, 1996) and increased secretion of digestive enzymes in parallel with increases in food consumption (Nitsan *et al.*, 1974). Finally there is some evidence that abalone alter their enzyme profile to cope with changes in diet (Erasmus, 1996; Fleming *et al.*, 1996; Knauer *et al.*, 1996; Taylor, 1994).

Many of the effects mentioned above have an impact on the intestinal surface area, digestive enzymes and transport proteins and hence digestive and uptake capacity of the intestine. Different energy sources may have non-starch polysaccharides that cause alterations to the length and weight of the intestine, disruption to the apical membrane of enterocytes, mucus environment and changes in the proliferative activity in the epithelial lineage of the mucosa (Brunsgaard and Eggum, 1995). With changes to the enterocyte apical membrane, nutrient digestion and transport will be greatly affected and hence there will be decreased nutrient uptake through the enterocyte. Damage to the apical membrane may also increase the permeability of the epithelium resulting in increased ion losses and associated transport activities. The mucus environment of an animal may change to such an extent that enteropathogens are able to adhere to the mucus more readily

and hence affect the health of the animal. In some cases digestibility of diets may or may not be greatly affected however the cost of repair to the intestine will reduce the available protein and energy for growth.

1.11 Summary.

This review has provided information about the abalone industry, concentrating on the nutritional aspects that are under consideration at present by researchers. The importance of the gastrointestinal tract has been highlighted as the major source of nutrient uptake and factors both developmental and nutritional that may affect efficient uptake. Due to a lack of knowledge of abalone, mammalian nutrient uptake mechanisms have been discussed, however, these have been limited to those that may apply to abalone based on common natural food sources and digestive capacities. The review also shows the previous work involved in understanding natural diet, feeding behaviour and enzyme profiles of the gastrointestinal tract as an aid in diet nutrient selection. The digestive enzyme profile of the intestine of abalone has not been adequately studied and results from such research may lead to improved diet formulation.

1.12 Potential Benefits of the Research.

The benefits of the present study will be the development of a more rapid assessment of nutrient source by using intestinal enzyme profiles. When several protein sources are used to ensure that the range of amino acids is supplied in diets, the effects may be seen using enzyme profiles rather than time-consuming growth trials. Although growth rates with diets give an indication of the suitability of a diet, the growth rates could be further improved by supplying a diet aimed at the profile of digestive enzymes. In abalone, a major difference to terrestrial farmed animals is the longer time required to reach marketable size. The use of histological techniques and isolated intestinal apical membrane to observe the effects which the diets or nutrient sources have on the structure and function of the intestine may provide additional information important to long term nutrition, growth and health of abalone. The techniques used in the present study might also be applicable to other marine and terrestrial species with a defined digestive system.

1.13 Objectives of the Research.

The major objectives of the research in this thesis are to:

1. Obtain a profile of wild reared and commercially reared abalone intestine structure and digestive enzymes.

2. Feed legume diets to commercially reared abalone and study the effects of anti-nutrient factors on the structure and function of the intestine.
3. Observe what interactions occur between the nutrient, diet and the apical membrane of the enterocytes of the intestine.
4. Observe what mechanisms are compromised in the intestine by feeding legumes to abalone.

The results of these studies will be discussed with implications to diet formulation, the consequences for digestion and uptake in the intestine of abalone, health and hence farmed abalone production efficiency.

Chapter 2

General Materials and Methods and Initial Experiments.

2. General Materials and Methods and Initial Experiments.

2.1 Aquarium System.

Abalone were housed in rectangular 10 litre acrylic aquaria. A flow through system was used with water filtered to a final 10μm nominal pore size by composite sand filters. The average water temperature was maintained at 18^0C. Aquaria were cleaned by siphon daily. Six to ten animals were housed in each aquarium dependant on the size of the abalone.

2.2 Abalone Tissue Sampling.

For the purpose of this study, the intestine (Plate 2.1) will be described as section 1 and section 2, based largely on the internal morphological changes that occur. Plate 2.2 shows the internal morphology of section 1 and section 2. The distal region of the intestine, referred to as the rectum, will not be included in this study as its morphology has the greatest density of mucus cells of the entire intestine. As such its suggested role (Campbell, 1965) is to 'package' the fecal pellets and hence should have little or no digestive or absorptive function.

Abalone were killed between 0800 and 1100 hours. The reason for this time period is that Britz *et al* (1996) found that carbohydrase and protease activity peaked at 12 – 18 hours after feed intake in abalone. Experimental samples were collected from the abalone intestine in two regions, the first at the proximal end of the intestine (denoted section 1 in this thesis) and the second (denoted section 2) just after a marked change in the intestine morphology where ridges seen in section 1 abruptly cease. The tissue was flushed with ice-cold phosphate buffered saline (pH 7.4) to remove digesta. Three abalone intestines had sufficient material set aside for use in histological and electron microscopy studies. For studies on mucosa histology a 10mm piece from each section of the small intestine was fixed in formalin for 24 hours and then stored in 70% ethanol. For electron microscopy studies, a 10mm piece from sections 1 and 2 was stored in a fixative: 4% paraformaldehyde, 1.25% Glutaraldehyde in phosphate buffered saline, 4% sucrose and 0.1% sodium azide. Usually, experimental numbers were 10 abalone of which the intestines of these 10 abalone were subsequently pooled to provide three replicates (2-3 abalone/replicate), weighed, wrapped in aluminium foil and snap-frozen in liquid nitrogen for use in the preparation of isolated apical membrane on which digestive enzyme functions were studied. The entire procedure from dissection to sample storage took between four and six minutes for each abalone.

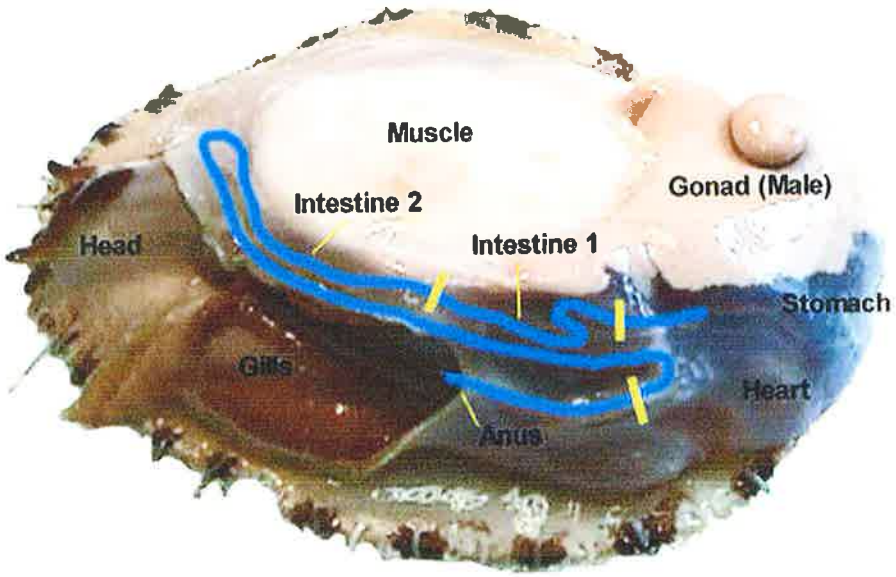


Plate 2.1: View of muscle and viscera (shell removed) of *Haliotis laevegata* with diagrammatic representation of intestine position.

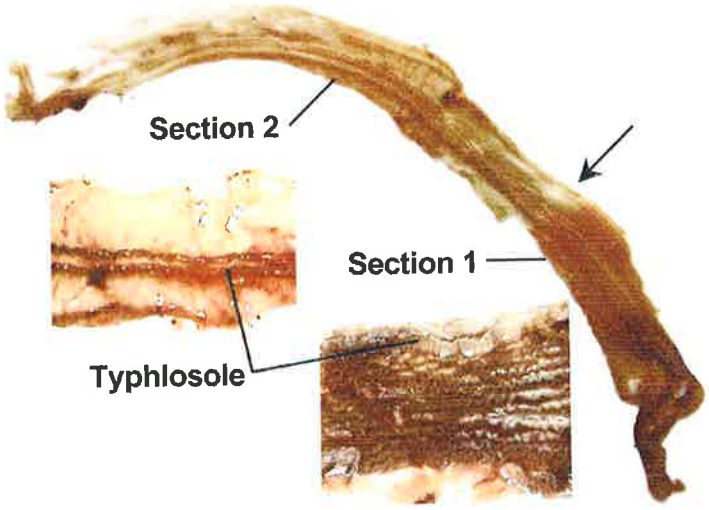


Plate 2.2: *Haliotis laevegata* Intestine: View showing sections 1 and 2 with magnified inserts. Note typhlosole that extends longitudinally throughout the intestine. Section 1 insert shows typhlosole and distinct longitudinal ridges. Arrow indicates abrupt change from section 1 to section 2.

2.3 Histology:

Slices of fixed tissues, each measuring 1-2 mm thick, were enclosed in tissue cassettes (Bayer Diagnostics, Aus. Pty Ltd). The tissues were processed over 16.5 hours in an automatic tissue processor (Shandon, Pittsburgh, USA). Processing consisted of serial dehydration with ethanol, clearing with histolene (Bayer Diagnostics, Aus. Pty Ltd) and impregnation with wax. The processed tissue was then embedded in paraffin wax. Sections were cut from the embedded tissue using a Leitz 1512 microtome (Ernst Leitz Westlar GmBH, Austria), cleared of surface undulations by floating on warm water (45-50°C) prior to mounting on 10 % poly-L-lysine coated slides.

2.3.1 Morphology and Mucin Assessment.

A series of sections were stained for tissue and nuclei by Lilee Meyer's hematoxylin, counter-stained with eosin yellow and mounted with DePeX medium (Bayer Diagnostics, Aus. Pty Ltd). Another series were stained for mucins using an alcian blue method counterstained with a periodic acid schiff reagent and mounted in DePeX medium.

2.3.2 Image Analysis.

Slides were viewed on an Olympus BH-2 microscope and digitised using the video image software, Video Pro (Leading Edge, Bedford Park, South Australia).

2.4 Electron Microscopy:

2.4.1 Scanning Electron Microscopy.

Dehydrated Specimens.

Fixed tissue samples were washed in phosphate buffered saline with 4% sucrose and postfixed in 1% Osmium tetroxide. Subsequent processing involved a serial dehydration in ethanol, placement in 100% acetone and drying in a critical point dryer. The dried tissue was subsequently mounted on stubs and coated with gold and carbon. Digitised images were obtained on a Philips XL30 field emission scanning electron microscope.

Hydrated Specimens.

In order to look at specimens in their native state a Phillips XL30 field emission scanning electron microscope was fitted with an Oxford CT1500HF Cryo Transfer system. The tissue was snap frozen in liquid nitrogen to ovoid the build up of ice crystals and transferred to the microscope using the Oxford system. Digitised images were then obtained.

2.4.2 Transmission Electron Microscopy.

Fixed tissue samples were washed in phosphate buffered saline with 4% sucrose and postfixed in 1% Osmium tetroxide. Samples were then serially dehydrated in ethanol and embed in araldite resin and polymerised in an oven at 60°C. Digitised images were obtained on a Philips CM100 transmission electron microscope.

2.5 Apical Membrane Preparation:

Intestinal membrane vesicles were prepared by the Mg^{2+} precipitation method as previously described by Sherazi-Beechey *et al.*, (1990). In brief, stored tissue (liquid nitrogen) was defrosted and homogenized in a hypotonic buffer [Mannitol 100mM, tris(hydroxymethyl)aminomethane (Tris) 2mM adjusted to pH7.1] to disrupt cell membranes. The resultant homogenate was filtered through a Buchner funnel (0.1mm) and a sample of this homogenate was taken for future enzyme analysis. $MgCl_2$ (10mM final concentration) was added to the homogenate as a precipitant (20 minutes) and the suspension was differentially centrifuged. Centrifugation was at 3000x g for 15 minutes with the resultant supernatant centrifuged at 30,000x g for 30 minutes. The resultant pellet was resuspended in a solution containing in mM Mannitol 100, Tris 2, $MgSO_4$ 0.1 pH7.4. This solution was centrifuged at 30,000 x g for 45 minutes and the final pellet resuspended in 1ml of storage buffer containing mannitol 300 mM, Tris 2 mM, $MgSO_4$ 0.1 mM and 0.02% NaN_3. The resultant apical membrane fraction and the homogenate were stored in liquid nitrogen for subsequent analysis.

2.6 Apical Membrane Enzyme Assays:

The selection of enzymes used in this study is based on the abalone natural diet of red algae, which is high in a Floridian starch similar to amylopectin. Enzymes that break down the products of amylytic hydrolysis of starch are the α-glucosidases maltase and sucrase. β-galactosidase was also used to test the ability of the abalone to hydrolyse β bonds, using lactose as a marker

substrate. Leucine amino-peptidase is a protease that is widespread in many terrestrial and marine animals and was thus included. No enzyme classification studies have been found to date that have isolated or proved that abalone have the particular enzymes mentioned above, however, the assays and substrates used in this study are those used in mammalian species. It can be said that the enzyme activities are, as an example, a "maltase-like" activity and in this study it will be referred to after the enzyme i.e. maltase activity. The units of enzyme activity in this study will be; specific activity (SA) (example maltase) μmole glucose/mg protein /hr while total activity (TA) is specific activity /gm tissue that will be designated μmole glucose/gm tissue.

2.6.1 Alkaline Phosphatase.

Alkaline Phosphatase was determined following protocols of Forstner *et al* (1968) against a known standard, 1μmole p-nitrophenol (Sigma). Assay conditions were a sample incubation period of 20 minutes at 21°C with a Sigma 104 phosphatase substrate in a 50mM tris(hydroxymethyl)aminomethane buffer pH10.1. Protein levels for the apical membrane fraction ranged between, 46 - 82μg and for the homogenate 62 - 122μg. The reaction was stopped with 40% Trichloroacetic acid, neutralised with sodium hydroxide and subsequent detection of p-nitrophenol by spectrophotometer at 410nm was obtained.

2.6.2 Carbohydrases.

Maltase, sucrase and β-galactosidase activity was determined following protocols of Dahlqvist (1968). Assay conditions were an incubation period of 30 minutes at 21°C with a 100mM maltose, sucrose or lactose substrate in a solution containing 4mM sodium succinate and 90mM NaCl ph6.0 or 100mM sodium citrate and 0.2mM *p*-chloromercuribenzoate. Protein levels for the apical membrane fraction ranged between 23 - 41μg and for the homogenate 31 - 61μg. The estimation of glucose released as a measure of enzyme activity was obtained using a Boehringer-Mannheim test kit (Chromogen - ABTS) measured at 610nm on a spectrophotometer.

2.6.3 Leucine Aminopeptidase.

Leucine Amino-peptidase activity was determined following protocols of Haase *et al* (1978) against a known standard Napthylamine (Sigma 251-10 1.8mg/100ml). Assay conditions were an incubation period of 30 minutes at 21°C with a 8mM leucine β-napthylamide substrate in a 200mM phosphate buffer pH 7.0. Protein levels for the apical membrane fraction ranged

between 0.92 – 1.63 μg and for the homogenate 0.31- 0.61 μg. The reaction was stopped with 32% Trichloroacetic acid and subsequent detection of Napthylamide was measured on a spectrophotometer at 560nm.

2.7. Intracellular Membranes:

2.7.1 Succinate dehydrogenase.

Succinate dehydrogenase activity was determined using protocols of Baginsky and Hatefi (1969). Protein levels for the apical membrane fraction ranged between 46 - 82μg and for the homogenate 62 - 122μg. Samples were pre-incubated for 10 minutes at 21°C in a solution containing 50mM potassium standard pH7.4, 20mM sodium succinate, 0.1mM Ethylene diamine tetra acetic acid and 0.1% bovine serum albumin. The reaction solution contained 70μM 2,6-dichlorindophenol, 1.65mM phenazine metho-sulphate and the pre-incubated samples. Succinate dehydrogenase activity was measured spectrophotomically by the phenazine metho-sulphate mediated reduction of 2,6-dicloroindophenol at 30-second intervals over 5 minutes.

2.7.2 Acid Phosphatase.

Acid Phosphatase was determined by using protocols of Freire *et al* (1995) against a known standard, 1μmole p-nitrophenol (Sigma). Assay conditions were an incubation period of 20 minutes at 21°C with a Sigma 104 phosphatase substrate in a 50mM tris(hydroxymethyl)aminomethane buffer pH10.1. Protein levels for apical membrane fraction ranged between, 46 - 82μg and for the homogenate 62 - 122μg. The reaction was stopped with 40% Trichloroacetic acid (TCA), neutralised with sodium hydroxide and subsequent detection of p-nitrophenol by spectrophotometer at 410nm was obtained.

2.8 Basolateral Membrane Sodium/Potassium ATPase:

Sodium/Potassium ATPase was determined using a combination of the methods of Quigley and Gotterer, (1969) and Qin *et al* (1992). Protein levels for apical membrane fraction ranged between, 46 - 82μg and for the homogenate 62 - 122μg. Samples were added to each of three solutions and incubated at 21°C for 5 minutes. The incubation solutions consisted of: Solution 1: 30mM tris(hydroxymethyl)aminomethane pH7.1 plus 7.5mM MgCl; Solution 2: solution 1 plus 120mM NaCl and 20mM KCl; and Solution 3: solution 2 plus 1mM ouabain. The assays were initiated by the addition of adenosine triphosphate (ATP) (5mM final concentration). Samples

were incubated for 30 minutes at 21°C and the reaction stopped with iced 40% Trichloroacetic acid. Samples were centrifuged at 2500x g with the supernatant removed for analysis of inorganic phosphate. The inorganic phosphorous produced was then measured colorimetrically by the Fiske and Subbarow procedure at 660nm on a spectrophotometer and the samples were measured against a phosphorous standard.

2.9 Protein:

Protein was determined using the methods of Bradford (1976) against a known concentration of Bovine Serum Albumin protein.

2.10 Initial Experiments:

2.10.1 Initial Isolation Procedure of Abalone Intestinal Apical Membranes.

The intestinal functional analysis required that the apical membrane of the abalone intestine epithelial cells be isolated. Wild- reared abalone were used in this study.

To assess the effectiveness of the isolation procedure, classical enzyme markers of cellular and intracellular membranes are determined. Based on the enrichment (ratio of specific activity of the enzyme in the apical membrane preparation to the specific activity of the enzyme in the crude homogenate) of the particular enzyme the 'purity' of the preparation obtained (Sachs *et al.*, 1980). The enrichments normally seen are, for enzyme markers of the apical membrane, greater than 1, for enzyme markers of intracellular and basolateral membranes, less than 1.

The purity of the apical membrane fractions from wild-reared abalone intestine (3 replicates of pooled abalone intestines, 30 individual abalone) showed enrichment (normally >1) for the markers of the apical membrane, alkaline phosphatase (2.9-3.2), leucine amino-peptidase (4.2-4.8), maltase (2.5-2.7) and sucrase (1.3-1.4). The marker of lysosomal membranes, acid phosphatase (normally <1) show enrichments of 0.50 - 0.62. Succinate dehydrogenase, a marker of mitochondrial membrane, was not detected in the apical membrane fractions and only trace amounts were detected in the homogenate. Na^+/K^+ ATPase, a marker of the basolateral cell membranes, (normally <1) was enriched 0.46 – 0.47. β-galactosidase showed an enrichment of

less than 1 (0.21 – 0.24). Protein recovery for the apical membrane fraction was 9.2% of the protein present in the original homogenate.

The apical membrane isolation procedure had been successful with results comparable to studies in mammals (Kessler *et al.*, 1978; Malo and Berteloot, 1991; Shirazi-Beechey *et al.*, 1990) and marine species (Reshkin and Ahearn, 1987) and it was assumed that the methods used would be suitable for studying the effects of dietary components on enzymes in this study.

2.10.2 Comparison of Intestinal Enzyme Activities in Wild-reared Abalone of sizes 55-70mm and 113-145mm.

This project is designed to be of practical application in abalone aquaculture as well as a scientific study as such the abalone obtained from the commercial farms are of sizes 45-85mm and are referred to as 'juvenile' abalone. In South Australia it is illegal to take wild abalone of sizes less than 113mm with this size abalone referred to as adults. The classifications of juvenile and adult may imply that the gastrointestinal tract is not at the same developmental stage and as such it was necessary to compare wild-reared abalone of juvenile (45-84mm) and adult size (>113mm). Permission was sought from the South Australian Fisheries to collect 20 undersize wild-reared abalone for this study. The aim was to prove that there was no difference in intestinal enzyme function between undersize and legal size abalone and as such legal sized abalone could be collected to provide the wild-reared abalone numbers for following experiments in the study.

The results are tabulated below and show that there were no significant differences at $P<0.05$ between the two ranges of size and as such legal size (>113mm) wild-reared abalone can be used for comparison to commercially reared abalone of the sizes mentioned.

Table 2.1: Comparison of Intestinal Enzyme Specific and Total Activities in the Homogenate and Apical Fractions of Intestinal Enterocytes in Wild-reared Abalone of sizes 55-70mm and 113-145mm.

Intestinal Enzymes		Wild-reared Abalone 45-70mm		Wild-reared Abalone 113-145mm	
		Homogenate	Apical	Homogenate	Apical
Alkaline Phosphatase	SA	1.70 ± 0.18	4.82 ± 0.59	1.70 ± 0.16	4.88 ± 0.62
	TA	24.82 ± 3.70	5.96 ± 0.68	24.14 ± 2.69	6.12 ± 0.69
Maltase	SA	1.62 ± 0.12	3.87 ± 0.31	1.57 ± 0.19	3.73 ± 0.29
	TA	24.17 ± 2.38	4.04 ± 0.11	24.00 ± 2.51	4.14 ± 0.19
Sucrase	SA	1.03 ± 0.02	1.30 ± 0.08	0.95 ± 0.09	1.25 ± 0.11
	TA	13.80 ± 1.37	1.49 ±0.09	13.59 ± 1.42	1.56 ± 0.13
β-galactosidase	SA	0.80 ± 0.06	0.16 ± 0.02	0.81 ± 0.07	0.16 ± 0.02
	TA	9.60 ± 0.71	0.17 ± 0.02	9.79 ± 0.84	0.18 ±0.02
Leucine amino-peptidase	SA	8.18 ± 0.52	29.1 ±0.60	8.30 ±0.47	29.60 ±0.71
	TA	94.65 ± 5.80	31.9 ± 1.12	98.9 ±4.80	33.90 ±1.27

Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity (SA) is expressed as μmole p-nitrophenol/mg protein/hr, total activity (TA) is expressed as μmole p-nitrophenol/gm tissue. Maltase, sucrase and β-galactosidase specific activity (SA) is expressed as μmole glucose/mg protein/hr, total activity (TA) is expressed as μmole glucose/gm tissue. Leucine amino-peptidase specific activity (SA) is expressed as μmole leucine-β-napthylamide/mg protein/hr, total activity (TA) is expressed as μmole leucine-β-napthylamide /gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment.

The results are not significantly different at $P<0.05$ for all tests. For Table clarity no denotation of significance is used.

Chapter 3

Impact of Rearing Regime on Intestinal Structure and Function in the Abalone *Haliotis laevegata.*

3. Impact of Rearing Regime on Intestinal Structure and Function in the Abalone *Haliotis laevegata.*

3.1 Abstract:

The digestive tract is an organ whose surfaces are constantly exposed to the rigors of the environment. Environmental effects, such as feed intake, type of feed and the products of digestion, may cause structural modifications to the digestive tract epithelia.

The structure and function of abalone intestine was investigated in wild (WRA) and commercially reared abalone (CRA), fed on two commercially available diets (CRA1 and CRA2), by histological and biochemical analysis.

The morphological changes seen in the intestinal mucosa, in abalone fed the commercial diets, compared to the features seen in wild caught abalone, generally included a combination of the following features:

1. The columnar cell nuclei are more centrally located,
2. An increase in the number of mucus cells,
3. In the area above the crypt region of the villus, non-vacuolated cells increase in number,
4. A profound infiltration of haemocytes in the lamina propria as well as interspersed in the columnar cells,

The biochemical analysis showed maltase to be the prominent carbohydrase in the abalone intestine. CRA1 had higher protease activity compared with CRA2. β-galactosidase activity was significantly higher in the CRA animals. The amount of protein/gm tissue in the intestinal apical membrane was significantly higher in the CRA animals.

The intestinal morphology of the CRA animals displayed characteristic features of an inflammatory response. In the same abalone, biochemical analysis of the intestine suggests inhibition of enzyme activity that may be caused by components of the protein and carbohydrate in the diet.

3.2 Introduction:

Domestication, farming, of Abalone imposes a significantly different rearing environment within which animals grow and develop, as compared with those raised in the wild. Generally for farmed aquatic species, diet type, feeding frequency, osmotic concentration of the rearing environment and social stress are known stressors that affect the digestive tract

(Nonette *et al.*, 1986). Of these, dietary effects can significantly alter the intestinal lining, thereby potentially affecting the digestive, absorptive, metabolic as well as the protective processes in any given individual.

The diet of abalone reared in the wild (WRA) consists mainly of macroalgae. The Australian abalone *Haliotis laevegata* prefers a diet of red algae (Rhodophyta), rather than brown algae (Phaeophyta), which is usually consumed by most other species of Australian abalone. Algal toughness (McShane *et al.*, 1994), phenolic content and the selective nature of environments on available alga (Shepard and Steinberg, 1992) are given as explanations of feeding preferences. *Haliotis laevegata* digest the less-preferred brown algae more slowly than red algae, indicating possible differences in digestive strategy to other abalone species (Foale and Day, 1992).

In abalone, information on the morphometry and morphology of the intestine is scarce. Previously, five regions to the intestine have been described, largely based on where contortions or directional changes were found. There are conflicting accounts as to the function of the intestine in abalone. The intestine of *Haliotis* is long, relative to body size, and provides ample surface for digestion and absorption according to Bevelander (1988). This contrasts to the function proposed by Campbell (1965), this being an area for organizing faecal rods. Harris *et al.* (1998c) found that the intestine has fewer cilia than the oesophagus, and suggested this may produce a slower food passage, which allows ample time for phagocytes to absorb food.

In terrestrial and marine animals, environmental stressors, most likely induced through variations in dietary intake, also affect the digestive tract. (Ehrlich *et al.*, 1976; Leeson *et al.*, 1992). Quality and quantity of ingested feed may cause structural modifications to the intestinal lining, especially the epithelial layer. Harris *et al.*, (1998c) studied the digestive tract of the *Haliotis laevegata* obtained from their natural habitat and characterised the intestinal structures in both fed and feed-restricted animals. Results, from their study, showed similarities to other marine and terrestrial species in that, the feed–restricted animals showed irregular arrangement of enterocyte nuclei, the apical vacuolated zone of cells was substituted by a finely granular cytoplasm and the occurrence of irregularly spaced indentations in the mucosa were also seen (Baeverfjord and Krogdahl, 1996). While this is informative, histological changes induced by removal of feed should be distinguished from

changes induced by feed and feed constituents, in particular those that are present in diets other than that found in the abalone natural environment.

Currently, fishmeal and soyflour are used as protein sources in most commercial abalone diets in Australia (Vanderpeer *et al.*, 1999). Being a terrestrial plant, soy may be an inappropriate feed component that is potentially damaging to the abalone intestinal structure and function. Digestive properties of the intestinal epithelium should be established, to see if there is a relationship between the intestinal morphology and impaired digestion due to the use of novel feedstuffs. Among the adverse effects seen in mammals are dramatic changes in structure, increased cell proliferation, excessive mucus formation and changes in enzyme activities within the small intestine (Govers *et al.*, 1993)

Most studies on abalone digestive enzymes have used the hepatopancreas or a combination of the crop and hepatopancreas. These studies have found abalone to have carbohydrate hydrolytic enzymes as well as proteolytic enzymes. Carbohydrases can be related to the structural polysaccharide linkages commonly encountered in natural diets (Nakada and Sweeny, 1967; McLean, 1970; Bennett *et al.*, 1971; Clark and Jowett, 1978; Boyen *et al.*, 1990; Knauer *et al.*, 1996). Herandez-Santoyo *et al.*, (1998) identified three serine-like proteases, (one chymotrypsin-like, two trypsin-like) as well as one carboxypeptidase in aqueous extracts from the hepatopancreas of *Haliotis fulgens*. Trypsin and chymotrypsin have also been indentified in the intestine of *Haliotis rufescens* and *Haliotis fulgens* (Groppe and Morse, 1993; Serviere-Zaragosa *et al.*, 1997). Serviere-Zaragoza *et al.*, (1997) found the highest proteolytic activity region to be in the intestine, with chymotrypsin 10 times higher than trypsin. This indicates a proactive role for the intestine in the digestion of feed in abalone. The fact that protease activity is greater in the intestine, as in mammals, may lead us to hypothesize that final carbohydrate digestion could also be found in the abalone intestine.

To assess intestinal digestive capability it is necessary to select enzymes that will provide an overview of the abalone digestion of feedstuffs in the intestine. In polar cells, such as those lining the intestine, there is a clear dichotomy of function with respect to the apical membranes (facing the lumen) and the basolateral membranes (in contact with blood) of the cells. The digestive enzymes are generally found in the apical region of the epithelial cells, as such it would convenient to use isolated apical membranes for testing purposes as problems related to metabolic degradation or binding of transported substances to intracellular structures is largely avoided. Isolation of this apical membrane is possible, but studies

investigating the impact of diet on enzyme activities have relied on biochemical analysis on a homogenate of the entire intestine tissue with concurrent histology of the villus crypt axis (Smith, 1990). Results being affected by the surface area to volume ratio of the tissue as well as the level of ingested protein absorbed through the vacuolated cells in the intestinal tract, make it difficult to assess the treatment effect in response to diet changes.

There were two objectives to the present study,

1. To distinguish characteristics of intestinal morphology related the rearing history of the abalone selected for study. A comparison was made between the intestinal structure of WRA and those observed in intestines of farmed abalone (CRA), fed two commercial abalone diets available in South Australia. Characterization was performed using histological sections and scanning and transmission electron microscopy.

2. To isolate the apical membrane of the intestinal epithelial cells using cation (magnesium) precipitation methods followed by an enzyme analysis of the area. WRA intestine was analysed to provide base line measurements of enzyme activity. Using the same commercial diets, as above, a comparison was made with WRA to see if differences could be found in enzyme activity due to diet and if so, how changes related to the observed morphology.

3.3 Methods:

3.3.1 Animals

The seventy-two CRA used in this experiment were from South Australian Mariculture Port Lincoln. Thirty-six WRA were collected from near Port Lincoln South Australia. Within each treatment group, nine of the abalone were used for histological studies and twenty-seven were used for biochemical analysis.

3.3.2 Diets and Feeding.

The commercial diets (1 and 2) were diets available in South Australia at the time of experimentation. Animals were fed the CRA diets to excess daily at 1700hrs for 14 days. Animals were fed to excess daily at 1700hrs for 14 days.

3.3.3 General Methods.

The general methods used in this study are detailed in Chapter 2 (General Materials and Methods), reference numbers are shown.

Abalone Tissue Sampling.	2.2
Histology.	2.3, 2.3.1, 2.3.2
Apical Membrane Preparation.	2.5
Electron Microscopy.	2.4.1, 2.4.2
Enzyme Assays	
Alkaline Phosphatase	2.6.1
Maltase, sucrase and β-galactosidase	2.6.2
Leucine Amino-peptidase	2.6.3
Succinate dehydrogenase	2.7.1
Acid Phosphatase	2.7.2
Sodium/potassium ATPase	2.8
Protein	2.9

3.3.4 Chemicals.

Glucose determination used a Boehringer-Mannheim test kit (Chromogen - ABTS) purchased from Adelab Adelaide, all other chemicals were the best available analytical grade and purchased from Sigma (Sydney Australia).

3.3.5 Statistical Analysis

The data are expressed as means ± SE. Significant differences between means were calculated with one-way analysis of variance on Genstat version 5 ($P<0.05$).

3.4 Results:

3.4.1 Morphological Studies:

General Description.

The gross morphology of the intestine shows three distinct sections. At the terminal part of the stomach there is a valve pointing into the intestine denoting the start of the intestine. Beyond, the first section of the intestine undergoes a convolution, after which the diameter is reduced and has little change until the rectum. The rectum is enlarged (width) in comparison to the previous section of the intestine. There is a double fold (typhlosole) that extends longitudinally throughout the extent of the intestine. For the purpose of this study, the intestine will be described as section 1, section 2 and the rectum (Plates 2.1 and 2.2 p34), based on the internal morphological changes that occur. Section 1 has a prominent typhosole and 11 simple folds, which are much shorter. This section begins to narrow, prior to the convolution, and continues until there is a distinct and abrupt change whereupon the folds

stop, leaving the typhlosole as the only visible feature. This marks the start of the second section of the intestine that continues until the rectum.

Light Microscopy:

Section 1 of the WRA intestine shows tall ciliated columnar cells, interspersed with mucus cells that line the mucosa of the intestine. Nuclei are oval and basally placed (Plate 3.1 top). The cytoplasm of the columnar cells is basophilic and occupied by translucent vacuoles of various sizes. The majority of cells in the crypt regions are non-vacuolated, show frequent mitotic figures, and have a finely granular homogeneous cytoplasm. Within the lamina propia, and interspersed among the epithelial cells, small populations of haemocytes are observed. Goblet cells are concentrated on the villus with few seen in the crypt regions. Section 2 of the WRA intestine again has the prominent typhlosole continuing however it is devoid of the 11 ridges seen in section 1. Columnar cells, interspersed with mucus cells, line the mucosa of this section of the intestine and show similar nuclei shape and location as in section 1.

The morphological differences seen to the intestinal mucosa (section 1), in the CRA animals, generally included a combination of features (Plate 3.1, middle and bottom):

1. The columnar cell nuclei are more centrally located,
2. An increase in the number of mucus cells,
3. On the simple villus structure, indentations can be seen with cells non-vacuolated in this area,
4. A profound infiltration of haemocytes in the lamina propria and interspersed in the columnar cells,

A shortening of the villus height and a thickening of the crypt was most notable in the CRA2 animals.

Several changes seen in section 2 of the CRA intestine are similar to those observed in section 1. These are the nuclei of the cells, which are more centrally located, there are indentations in the epithelial layer and more haemocytes are seen. In the CRA fed commercial diet 1 (CRA1), the lamina propria is much thicker. Secretory cells are also more noticeable in CRA fed commercial diet 2 (CRA2), while granulated cells are more prominent in the CRA1.

Ultra-Structure Studies:

Surface Characteristics:

There is a clear difference between mucus covering the surface of the intestine of the WRA group compared with the CRA group. On the upper region of section 1on the WRA intestinal

villus structure (Plate 3.2 a), there are several fine, but open, mucus layers which allows underlying structures to be seen, while the CRA1 (Plate 3.2 b) and CRA2 (Plate 3.2 c) groups have much thicker layers. The CRA1 group mucus cover consists of many layers made up of fine strands that form together to give a sponge-like appearance. Similarly layered mucus is also seen in the CRA2 group, however the strands are wider producing a more sheet like appearance.

In the crypt regions, the mucus covering is thicker in all diets. The WRA intestine (Plate 3.3 a) has many layers of interwoven fine mucus strands forming a sponge-like appearance, while the CRA2 group (Plate 3.3 c) is a distinct sheet. The CRA1 intestine (Plate 3.3 b) has various width mucus strands, which are, mainly, much wider than those observed in the WRA group. The wider strands form into small sheets, however the spaces between these strands are larger than those seen in the top sections of abalone fed this particular diet.

Epithelium Apical Region.

The fixation and dehydration of the tissue has largely removed the mucus layer and allowed the underlying structures to be seen. All abalone intestines show similar features, the most prominent being the spherules (Plate 3.4 a) that cover the majority of the surface of the villus. There are areas where no spherules are seen, however there are indications that they have been discarded from the top of the epithelial cells. Where these spherules are missing, the cilia can be seen clearly. They appear largely around the top margins of the epithelial cells rather than completely covering the cells. The spherules are attached to the cell by a fine thread structure that protrudes from the top of the epithelial cell.

In the CRA group prominent secretory cells protruding above the other cells into the lumen are seen in the centre of the typhlosole (Plate 3.4 b CRA2) and in the crypt region. Globules of secretory product can be seen within the secretory cells and, in this study, a number of cells showed release of these products from the top of the cells after forming a small circular opening.

Sub-Cellular Structures.

WRA intestinal (Plate 3.5 a) columnar cells show numerous lysosomes in the apical region. The cells are highly vacuolated, and have prominent cilia and cell boundaries as well as rough endoplasmic reticulum, mitochondria and ribosomes. The CRA groups (Plate 3.5 b, c) have less vacuoles and lysosomes. Haemocytes appear in the apical region among the columnar

cells more frequently in the commercial diets, as does the number of secretory cells. These cells differ from goblet cells and can be distinguished by the secretory globules, which are approximately twice the size compared to those seen in the goblet cells. The CRA2 group (Plate 3.5 c) shows an abundance of secretory cells.

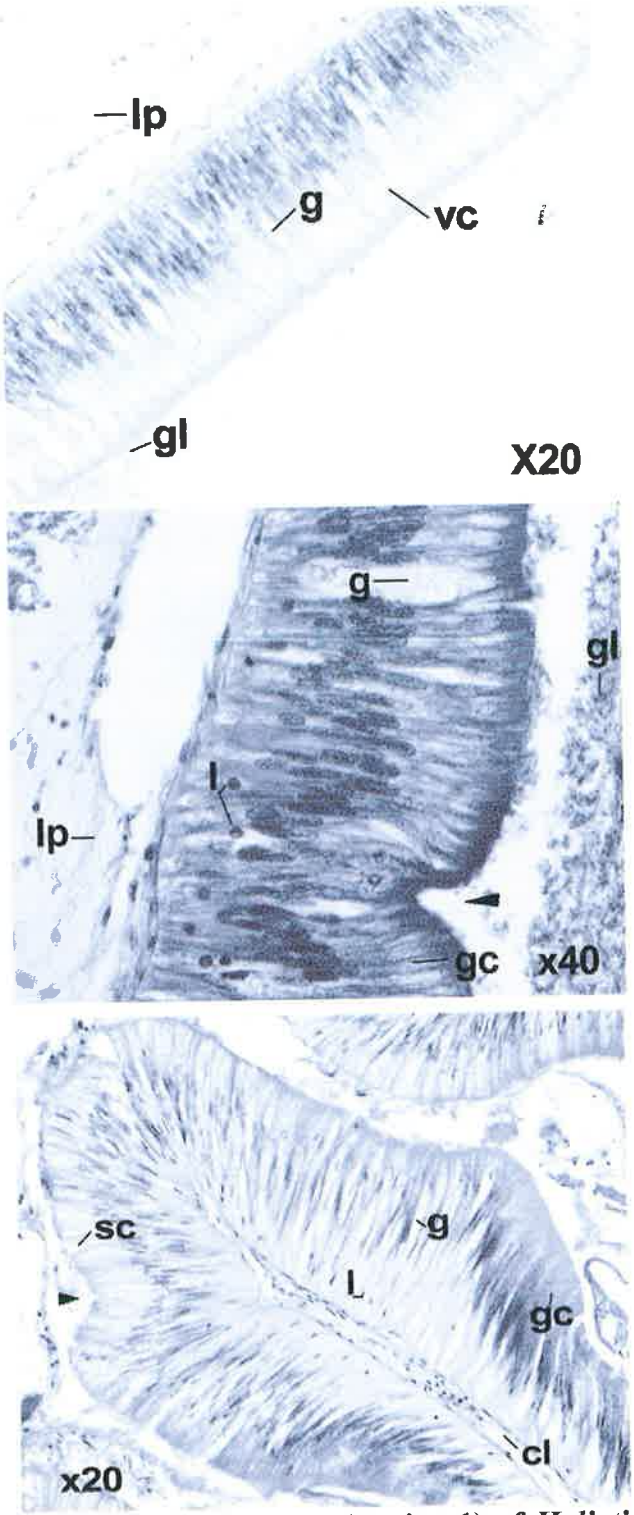


Plate 3.1: Detail of intestinal epithelial cells (section 1) of *Haliotis laevegata* in Wild-reared Abalone (top), Commercially Reared Abalone fed commercial diet 1(middle), Commercially Reared Abalone fed commercial diet 2 (bottom). Note basally located nuclei in wild-reared abalone and nuclei more centrally located in commercially reared abalone intestine. Increased numbers of haemocytes are seen in the lamina propria and interspersed in the columnar cells in the commercially reared abalone intestine. Increased numbers of granulated cells are seen in the commercially reared abalone intestine. cl = central lacteal, g = goblet cell, gc = granulated cell, gl = glycocalyx, l = haemocyte, lp = lamina propria, sc = secretory cell, vc = vacuolated cell.

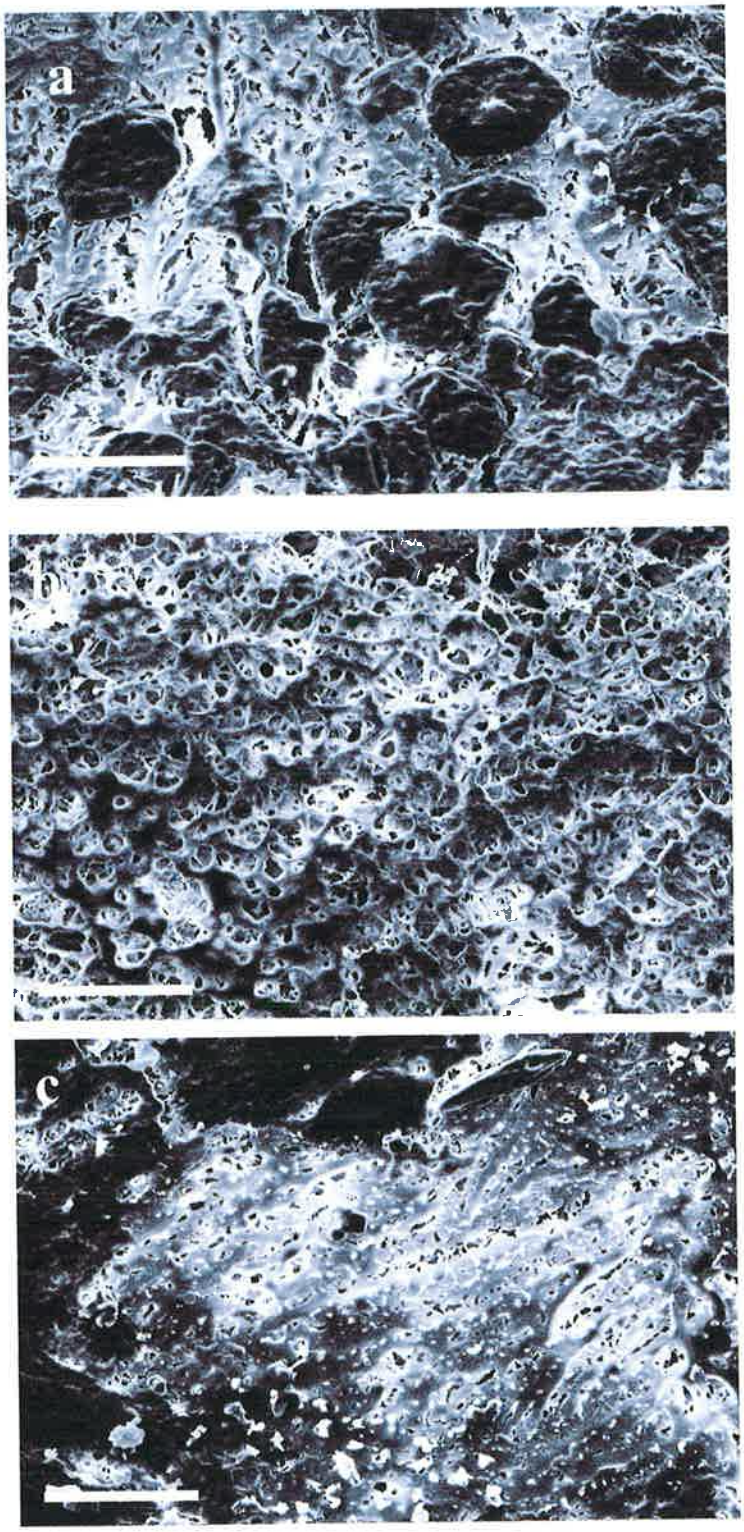


Plate 3.2: Epithelial surface of the abalone *Haliotis laevegata* intestine section 1 villus top showing surface mucus coating using SEM fitted with a cryo transfer system. (a) Wild – reared abalone, (b) Commercially reared abalone fed diet 1, (c) commercially reared abalone fed diet 2. Bar =10μm. Note open mucus layer of wild –reared abalone allowing underlying structures to be seen compared with much thicker layers in the commercially reared abalone.

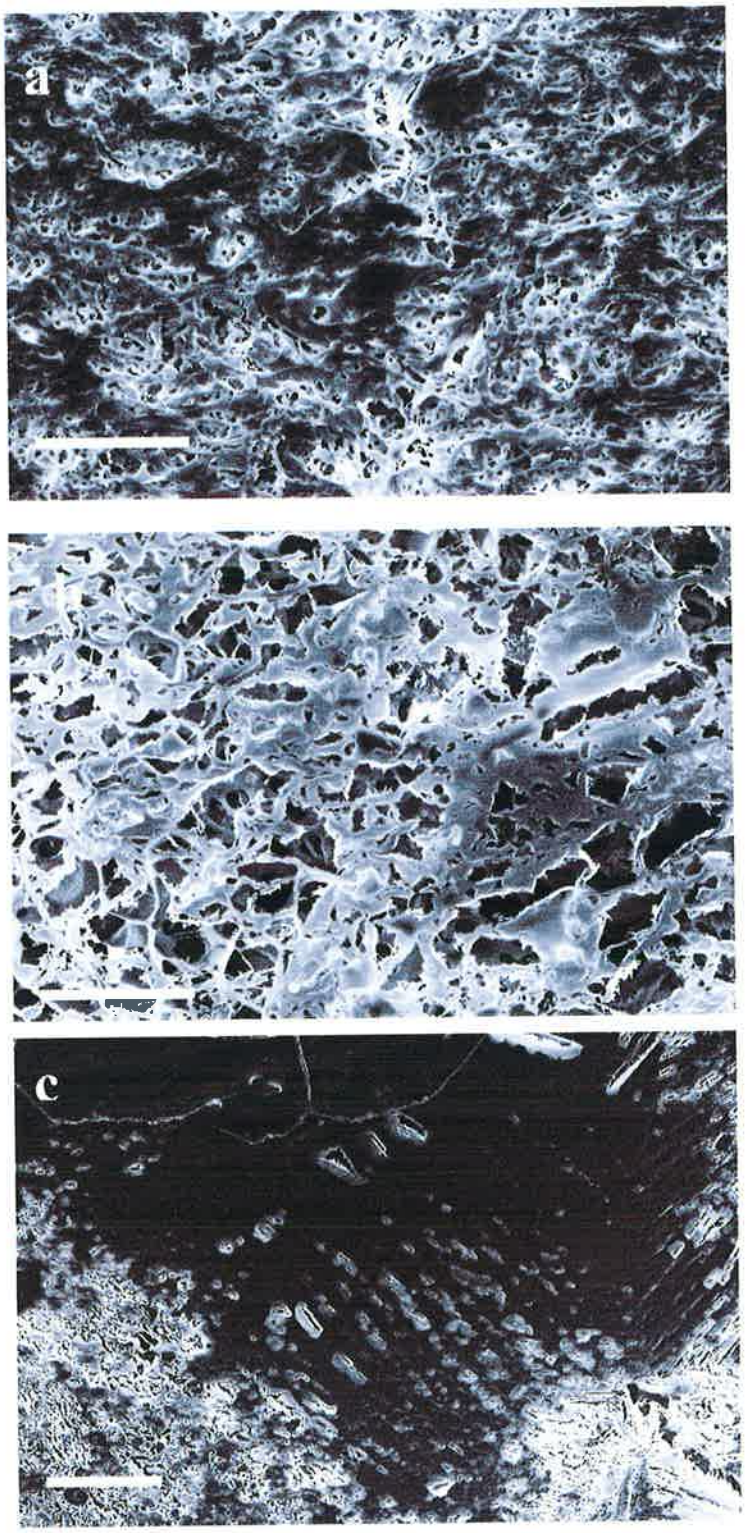


Plate 3.3: Epithelial surface of the abalone *Haliotis laevegata* intestine section 1 crypt region showing surface mucus coating using SEM fitted with a cryo transfer system. (a) wild-reared abalone, (b) commercially reared abalone fed diet 1, (c) commercially reared abalone fed diet 2. Bar =10μm (a&b) Bar = 50μm (c). Note dense coating of mucus in the intestines of wild-reared abalone and commercially reared abalone fed diet 1 and the thick flat sheet appearance of commercially reared abalone fed diet 2.

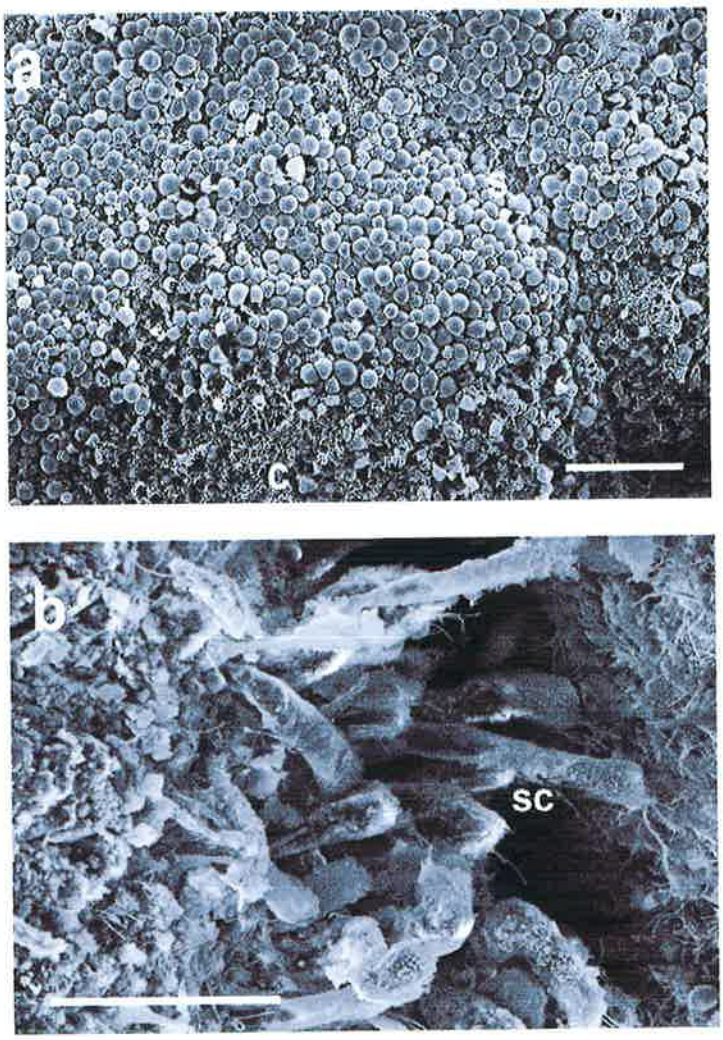


Plate 3.4: Epithelial surface of the intestinal villus (section 1) of *Haliotis laevegata* showing epithelial cells covered by spherules (a) and secretory cells (b) are seen projecting past the apical regions of the epithelial cells in the intestine of commercially reared abalone fed diet 2 (b). Bar = 50μm (a) and 20μm (b). c = cilia, s = spherules, sc = secretory cells.

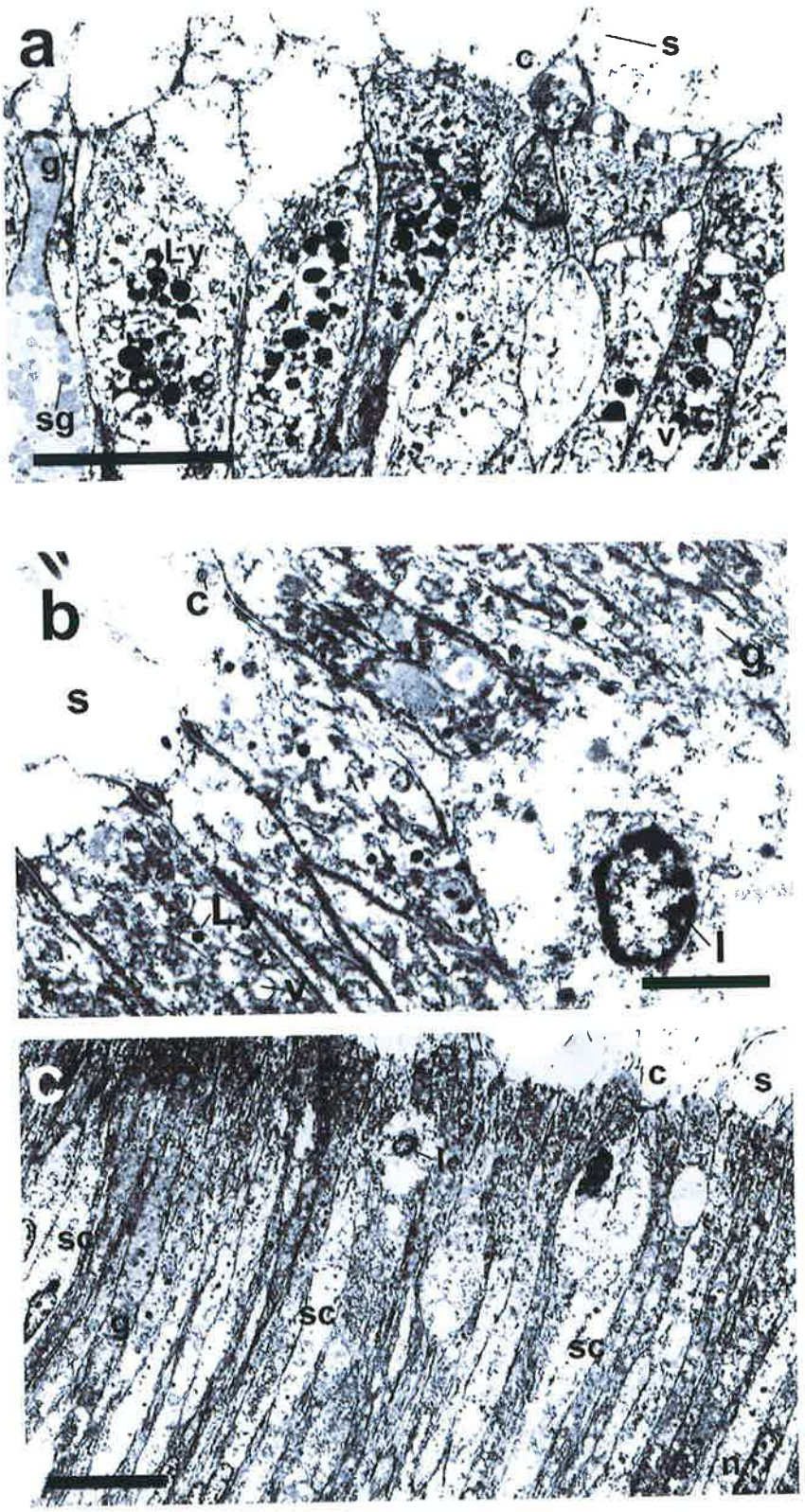


Plate 3.5: Transmission Electron Microscopy showing portion of *Haliotis laevegata* intestinal mucosa section 1. (a) wild-reared abalone, (b) commercially reared abalone fed diet 1, (c) commercially reared abalone fed diet 2. Bar = 10μm (a, c) and 5μm (b). Note in the wild-reared abalone the numerous lysosomes and the highly vacuolated cells that are not as prevalent in the commercially reared abalone. In commercially reared abalone fed diet 2 there is an additional secretory cell that is easily distinguished from a goblet cell. c = cilia, g = goblet cell, ly = lysosome, l = haemocyte, n = nucleus, s = spherule, sc = secretory cell, sg = secretory globule, v = vacuole.

3.4.2 Functional Characteristics:

Apical Membrane Isolation.

The apical membrane isolation procedure had been successful and would be suitable for studying the effects of dietary components on enzymes in this study. Protein recovery for the apical membrane fraction was between 8.9% and 9.8% of the protein present in the original homogenate.

Functional Capabilities, Digestive Enzymes.

Alkaline Phosphatase.

There were no significant differences between diet treatments for specific activity (Fig 3.1). However, CRA2 shows a total activity/gm tissue more than double the value of other diets.

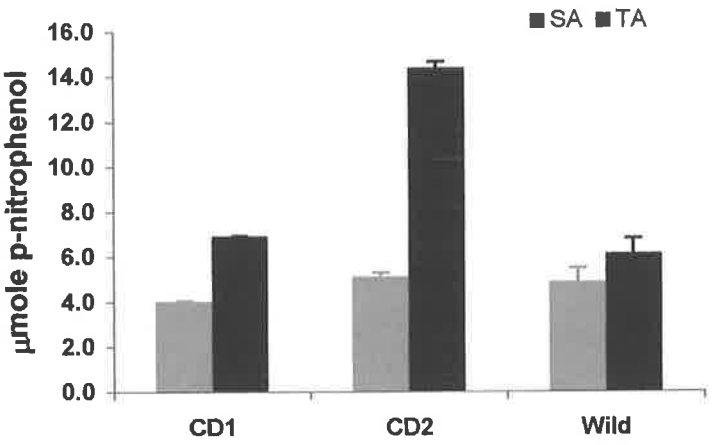


Figure 3.1: Effect of Diet on Intestinal Alkaline Phosphatase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.
Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity (SA) is expressed as µmole p-nitrophenol/mg protein/hr, total activity (TA) is expressed as µmole p-nitrophenol/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. TA of CD1 and wild was significantly different to the TA of CD2 at $P<0.05$. Commercial diet 1 (CD1), commercial diet 2 (CD2), wild-reared abalone intestine (wild).

Carbohydrases.

Carbohydrase activities were expressed in the intestine with maltase > sucrase> β-galactosidase (Figure 3.2). The WRA intestine has higher specific activities in maltase (Figure 3.2a) and sucrase (Figure 3.2b) compared with the CRA intestines. However, β-galactosidase (Figure 3.2c) is significantly lower in the WRA intestine than in the CRA groups. The total activity in the intestinal apical membrane is significantly higher in the CRA groups, than in the WRA group, for maltase (Figure 3.2a) with the reverse being true for sucrase (Figure 3.2b). β-galactosidase total activity is significantly higher in the intestine of the CRA groups compared with the WRA group.

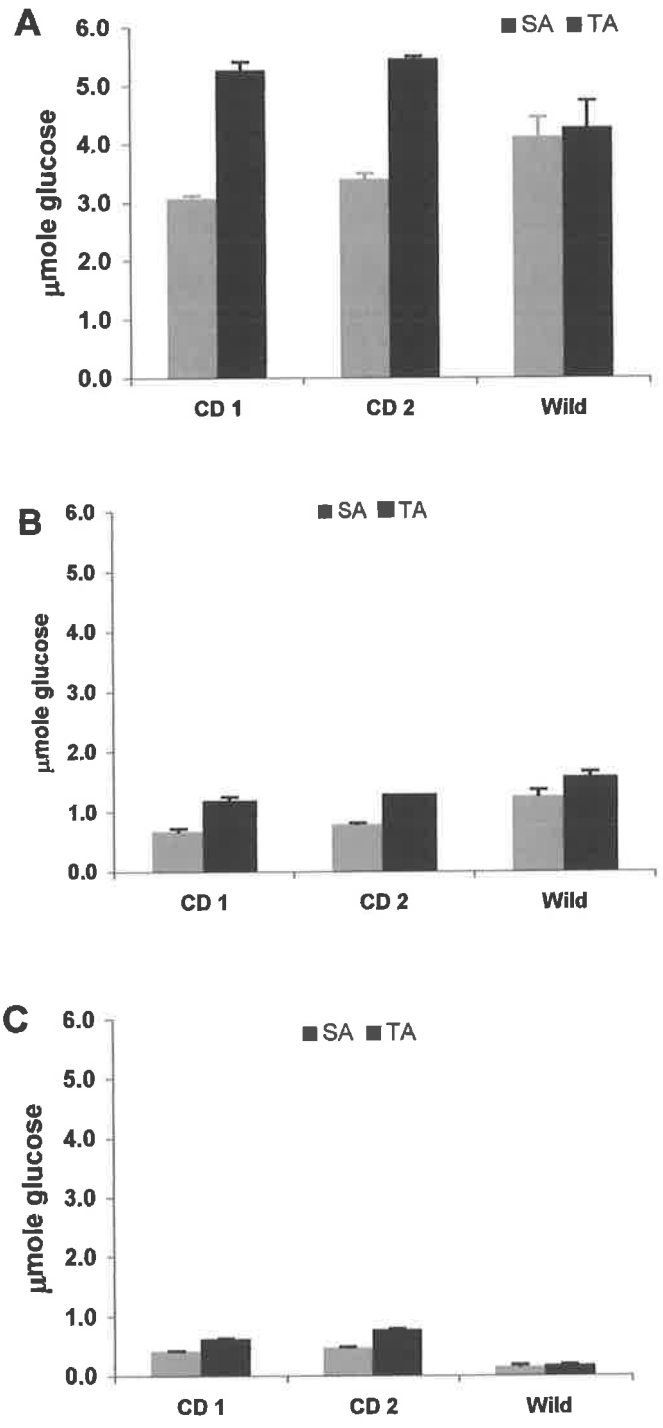


Figure 3.2: Effect of Diet on Intestinal Carbohydrases Maltase (A), Sucrase (B) and β-galactosidase (C) Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole glucose/mg protein/hr, total activity (TA) is expressed as µmole glucose/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. For maltase and sucrase SA and TA for CD1 And CD2 were significantly different to wild at $P<0.05$. For β-galactosidase all intestinal apical membrane SA and TA for each diet treatment were significantly different at $P<0.05$. Commercial diet 1 (CD1), commercial diet 2 (CD2), wild-reared abalone intestine (wild).

Protease.

The apical membrane marker leucine amino-peptidase specific activity and total activity in the apical membranes is significantly higher in the CRA1 group than in the other two rearing regimes (Figure 3.3). The specific activity of intestines from the CRA2 group is significantly lower than other diet regimes.

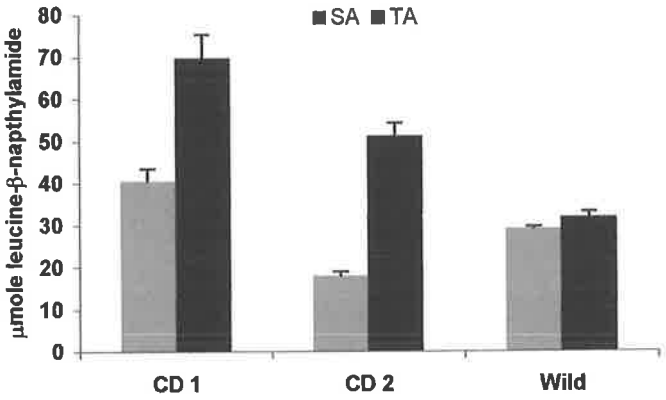


Figure 3.3: Effect of Diet on the Protease Leucine amino-peptidase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole leucine-β-napthylamide/mg protein/hr, total activity (TA) is expressed as µmole leucine-β-napthylamide /gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. All intestinal apical membrane SA and TA for each diet treatment were significantly different at $P<0.05$. Commercial diet 1 (CD1), commercial diet 2 (CD2), wild-reared abalone intestine (wild).

Total protein/gm tissue in the intestinal apical membrane fractions, for the CRA groups is significantly greater than those from the WRA group (Figure 3.4).

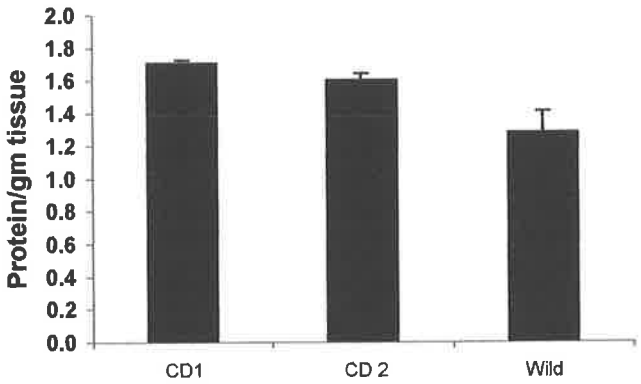


Figure 3.4: Effect of Diet on the Amount of Protein per gram Tissue in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Single factor ANOVA was used to assess significant differences for diet treatment. Protein/gm tissue for CD1 and CD2 were significantly different to wild at $P<0.05$. Commercial diet 1 (CD1), commercial diet 2 (CD2), wild-reared abalone intestine (wild).

3.5 Discussion:

3.5.1 Morphological Studies:

In WRA the intestinal epithelium is typical of an epithelium seen in terrestrial animals and fish with the exception of the lack of apical membrane microvilli. Instead club shaped spherules protrude from the apical membrane of digestive cells with cilia seen on the outer edges of the cells. Morton (1953) suggested the term 'fragmentation phagocytes' in describing the spherules. Morton postulated their function to be a replacement for amoebocytes whereby the digestive cells bulge to form the club shape, detach, nucleus included, moving through the gut, secreting digestive enzymes. Harris *et al.*, (1998c) suggests their role to be either waste removal or secreting digestive enzymes. In the present study, spherules provided a complete coverage of the villus on all samples, including those where no food was present in the intestine. This suggests that they are not cyclic as expected if their role was to detach to secrete digestive enzymes in the lumen or provide a waste removal role. No evidence that the spherules contained intracellular organelles or nuclei was seen on any samples in this present study. In mammalian intestine microvillus structures contain digestive and transport enzymes. In samples studied by transmission electron microscopy (TEM), the spherules show a fine membrane that can be seen in cross-section. Attached to this membrane are distinct figures that may be protein structures or areas of endocytosis of food particles. It may be postulated that these spherules are a primitive form of the microvillus and provide the additional surface area for digestive and absorptive function.

The morphology of the intestine in the CRA displayed characteristic features of inflammation. The lamina propria and the epithelial cell layer were heavily infiltrated with haemocytes. Granulated cells in the crypt areas increased in number and their distribution increased to include more of the lower villus. Goblet cells increased in number, particularly in the crypt regions, as well as other secretory cells. The mucus layer of the intestine is much thicker and denser in the CRA groups than in the WRA, indicating increased mucin secretion as well as increased polymerisation of the mucin monomers. While no studies have looked at potential allergens in abalone, it seems appropriate to suggest that in the CRA a non-infectious subacute enteritis with a pathogenesis involving immunological mechanisms may be the reason for the morphological changes seen in the intestines in this study. These features may be used as markers in future studies to identify abalone that have compromised intestinal function. A relationship has been found between an intestinal pathology, similar to that seen

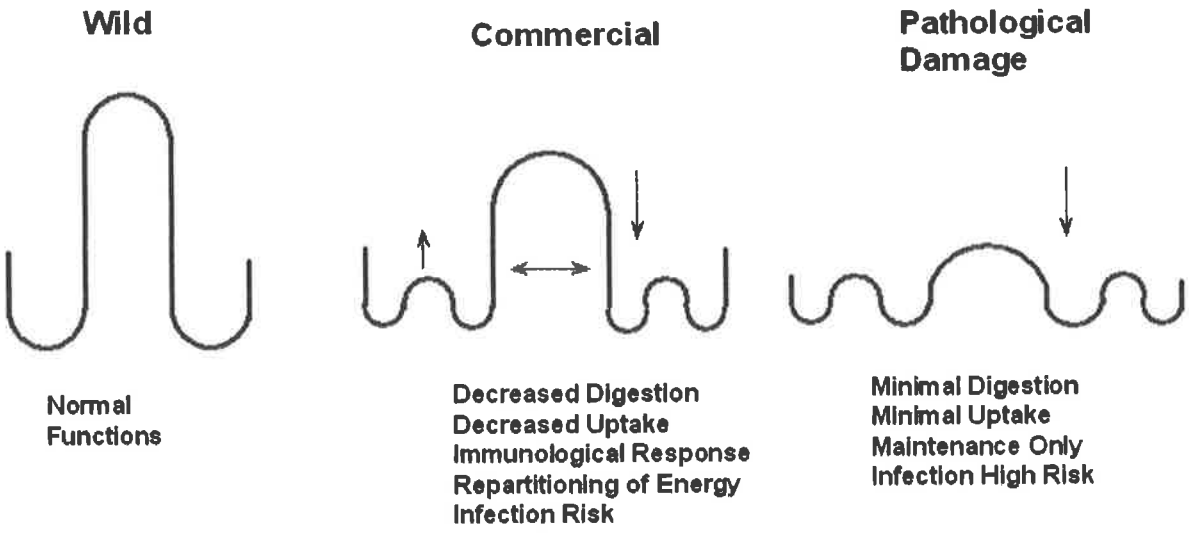
in this study, and impaired nutrient uptake and digestion in rainbow trout (Rumsey *et al.*, 1994) and in Atlantic salmon (Baeverfjord & Krogdahl 1996).

The morphological alterations seen in this study are consistent with reports in marine and terrestrial animals where the inclusion of soybean, as an alternative protein source, has repeatedly been shown to cause adverse effects on the intestinal structure (Baeverfjord and Krogdahl, 1996). The problems related to soybean use in diets have been ascribed to the presence of antinutrient factors (ANFs) including lectins, protease inhibitors as well as allergenic proteins (Liener, 1994). Lectins have been shown to cause hyperplasia and hypertrophy in rat small intestine (Pusztai, 1989) and adverse effects on enterocyte viability and absorptive function (Hisayasa *et al.*, 1992). Morphological alterations to the small intestine of mammals has been reported, after feeding soybean, including shortening of villus height and an increase in crypt depth (Dunsford *et al.*, 1989; Li *et al.*, 1991; Ratcliffe *et al.*, 1989; Silva *et al.*, 1986). A shortening of the villus height and a thickening of the crypt is most notable in CRA2. Diets that are protein deficient can also show these effects on the intestine structure (King *et al.*, 1983; Smith and Peacock, 1989; Syme and Smith, 1982). Protease inhibitors may cause protein deficiencies by forming chelates with the enzyme reducing its activity (Petterson and MacIntosh, 1994). Allergenic proteins can create an inflammatory response that can also cause chronic changes to the intestinal tract.

The intestine surveys antigens in the intestinal lumen by allowing some to permeate the epithelium and interact with the intestinal and systemic immune systems (Ciancio and Chang, 1992). However excessive or inappropriate exposure of antigens to the intestinal immune system may lead to gastrointestinal disease (Walker and Sanderson, 1992). Physiologic and pathologic stimuli including allergenic proteins, inflammatory mediators and bacterial toxins accelerate intestinal mucus secretions, forming highly cross-linked aggregates with the physical properties of a gel. This mucus layer can impede the diffusion of noxious agents however still allow diffusion of low molecular weight solutes (Lamont, 1992). With repeated mucosal injury, the number of well differentiated absorptive cells decreases and the number of crypt, regenerative cells increases, resulting in a 'leakier' dysfunctional state in the intestine (Ciancio and Chang, 1992). The intensity of the immunoregulation is enhanced with significant increases in inflammatory cells in the lamina propria as a result of mediator-induced chemotaxis and increased vascular permeability (Ciancio and Chang, 1992; Madara *et al.*, 1992). Increased numbers of fibroblasts, polymorphonuclear cells, macrophages, mast cells, eosinophils and lymphocytes are typically seen in this area (Baeverfjord and Krogdahl,

1996; Ciancio and Chang, 1992; Rumsey *et al.*, 1993). These features resemble conditions like coeliac disease (Crawford, 1994), caused by an allergic response to gluten proteins, and inflammatory bowel disease (Ciancio and Chang, 1992) in humans and are typical of an intestinal inflammatory response.

The figure below shows a possible scenario if the intestine is subjected to repeated mucosal injury. If the intestine is compromised then it is more likely to be effected by challenges such as bacterial or viral attack, toxic components of diets or factors that may inhibit digestive enzyme function and hence uptake of nutrients.



Vacuolated epithelial cells in this study were reduced in number in both CRA intestines, being replaced with highly granulated cells. Harris *et al.*, (1998c) in their study, which looked at fed and feed-restricted WRA, show that in fed abalone the nucleus was basely located in the cells and in feed-restricted animals the nuclei were located more centrally. They also found epithelial cells in feed-restricted animals became more granular and the villus structure to be more irregularly shaped. These features were noted in this study, however, the granulated cells were mainly in the lower region of the villus with vacuolated cells present on the tip regions of the villus. From this information it suggests that the changes are closely related to dietary contents, therefore the likelihood of an infectious aetiology seems negligible.

3.5.2 Functional Analysis:

The functional properties of the cell can be affected by products of digestion, in particular the antinutrient factors that may interact with the apical membrane receptors and enzymes.

- The effects on enzyme function can be either reversible or irreversible. The former involves non-covalent binding of the inhibitor and can be reversed by removal of the inhibitor. In irreversible inhibition a molecule is covalently bound to the enzyme and incapacitates it. Reversible inhibition can take two forms, competitive inhibition and non competitive. Competitive is where a molecule that closely resembles the substrate is accepted into the catalytic site of the enzyme, reducing its effectiveness. Non-competitive inhibitors attach to a site on the enzyme that is not the catalytic site and modify the shape of the enzyme such that the effectiveness of the enzyme is reduced. Effectively there is an inhibition of the enzyme function.

- Another response is to stimulate the synthesis of the enzyme causing an increase in enzyme protein in the apical membrane. A change in the number and type of cell can also occur with stimulation from feedstuffs such as an increase in mucus or secretory cells.

- The enzyme specific and total activities may change with the effects mentioned above, i.e. if an enzyme has been inhibited then the specific activity may be reduced which may result in a deficiency of nutrients. The animal's response may be to increase the synthesis of the proteins such that there is enough enzyme activity to gain those necessary nutrients. In this case the specific activity decreases and the total activity increases.

- If the enzyme is not inhibited and there is little nutrient in the intestine the animal may again increase the specific activity and increase the synthesis of the enzyme protein placing more enzyme protein in the apical membrane. Here the specific and total activities would increase.

- Should there be sufficient nutrients available in the intestine then the specific activity of the enzyme may reduce and there may also be less requirement for the amount of enzyme protein to be in the apical membrane, resulting in a decrease in both enzyme specific and total activity.

As an example, and as a summary of the specific and total activity of enzymes seen in the CRA, enzyme activity in CRA relative to the activity found in WRA is shown in figure 3.5. The specific and total activity of the enzymes in WRA have been normalized to one, with the CRA enzymes shown either as a relative decrease or increase compared to the WRA. Considering CRA maltase activity, the specific activity is lower than the WRA and the total activity is higher, implying there is both an inhibition of the enzyme and an increase in synthesis of the enzyme protein providing increased enzyme protein in the tissue. CRA β-galactosidase shows an increase in both activities indicating a more active enzyme and more enzyme protein in the apical membrane. CRA sucrase activities show both reduced, which could be an inhibition of the enzyme or simply there is little requirement for this enzyme in the final digestion of these diets.

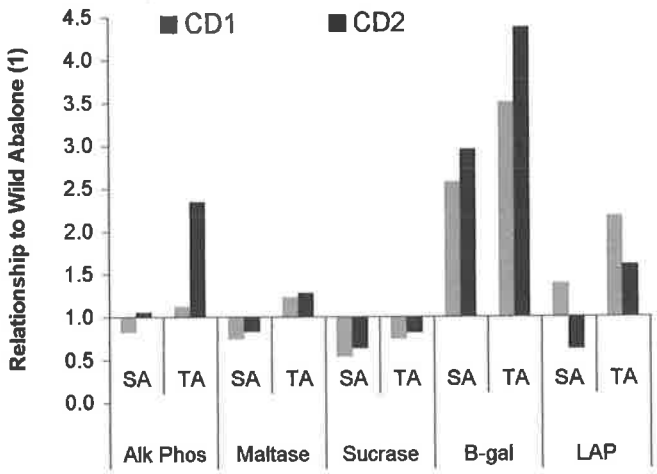


Figure 3.5: Specific and Total Activities of Commercially Reared Abalone Intestinal Enzymes Relative to Wild Abalone (*Haliotis leavegata*) Intestinal Enzymes.
SA and TA denote specific activity and total activity respectively of an enzyme. Alk Phos is the enzyme alkaline phosphatase, B-gal is b-galactosidase and LAP is leucine amino peptidase. CD1 and CD2 relates to two different commercial abalone diets.

Alkaline phosphatase

In this study, alkaline phosphatase specific activity did not vary with rearing regimes. The significance and exact role/s of membrane bound alkaline phosphatase in enterocytes is still being established. A known function is to dephosphorylate molecules, liberating inorganic phosphate (Mizuno *et al.*, 1992). Some properties that have been postulated for alkaline phosphatase are its dependency on zinc for activation (Yamamoto *et al.*, 1991). It is also inhibited by cysteine, mucin, lectins and dextran sulphates (Mizuno *et al.*, 1992). The most notable result in this study was the total activity/gm tissue of the CRA2 group, which was more than double that of all other abalone. These animals may have been synthesising more

alkaline phosphatase enzyme to liberate inorganic phosphate needed for normal functioning of the epithelial cells membranes and/or metabolic processes. Several factors may be involved in this response to a reduced phosphorous level including inhibition of the alkaline phosphatase enzyme by lectins as noted by Mizuno (1992) or a decreased availability of phosphorous caused by Phytic acid in plants binding phosphorous rendering it indigestible (Liener1994). Alkaline phosphatase specific activity has been shown to increase in as little as 18-24 hours with deprivation of food and substantially increase with starvation (Bamba *et al.*, 1990; Majumbar and Panda, 1989; Palmer and Rolls, 1983). The histology of the CRA2 group showed increased granulated cells similar to that of tissue obtained from feed-restricted animals (Harris *et al.*, 1998c) and may be the reason for the increase in alkaline phosphatase activity.

Carbohydrase activity:

The high level of maltase activity reflects the need to breakdown maltose liberated by amylytic hydrolysis of starch. The high maltase activity observed in the intestinal tract, compared with other disaccharidases, is in agreement with other aquatic species, Rainbow Trout, Dover Sole, Halibut and Atlantic Salmon (Buddington and Diamond, 1987; Clark *et al.*, 1984; Glass *et al.*, 1987; Krogdahl *et al.*, 1999).

β-galactosidase was tested to see to what extent abalone could hydrolyse β-bonds. Very little activity was observed. The enrichment of β-galactosidase was <1, which indicates that the enzyme is concentrated in areas other than at the apical membrane. Its role may be related to the hydrolysis of galactosides, which are abundant in the intestine and present in mucosal cells, secreta and micro organisms (Allen, 1981). The CRA group showed a significant increase in comparison to the WRA group. The β-galactosidase activity may, therefore, have been non-specific galactosidase activity present in the cytosol, bound to non-apical membranes or to galactosides in mucus cells. The increase in mucus cells was greater in the CRA2 group than in CRA1 group as was the increase in β-galactosidase.

Maltase and sucrase specific activities in the CRA intestine were significantly lower than in the WRA and the enrichments showed that the activities for sucrase was mainly intracellular as was maltase in the CRA1 group. The lower disaccharidase activity seen in the CRA compared with WRA may reflect the type of starch and how well it has been hydrolysed in previous sections of the gut. Semolina is used in commercial diets in Australia and has a more complex structure than the red algae consumed by WRA (Vanderpeer *et al.*, 1998). There may

also be an effect from antinutrient factors in the commercial diets. Several studies have shown that limiting feed, or feed-restricting animals, can reduce the activity of maltase and sucrase and is most likely due to a real decline in enzyme contents rather than modifying the enzyme activation (Holt and Yeh, 1992; Krogdahl *et al.*, 1999; Nakata and Kimura, 1989).

In the present study, the specific activity of the carbohydrases was reduced in the CRA group compared with the WRA group however the total activity was increased in the tissue. A component of soyflour is lectins which have multiple binding sites and can bind to membrane receptors of epithelial cells, cross link to each other forming a membrane or cell agglutination resulting in a reduction in the efficiency of nutrient digestion and absorption (Irish *et al.*, 1999). The response of the CRA groups for the increase in total activity may be to increase the synthesis of enzyme protein to counteract the loss in activity.

Protease activity:

Leucine amino-peptidase activity is mainly in the apical membrane. CRA2 shows the lowest specific activity and is most likely due to inhibition or the additional mucins that are seen with this diet, masking the activity. The difference seen in the total activity of the apical membrane between the WRA and CRA groups may reflect the additional protein content of the commercial diets (28%) as compared to 17% protein found in algae (Foale and Day, 1992). The low leucine amino-peptidase specific activity for CRA2 may reflect what was seen in the histological study in that the tissue resembled that of a protein deficient or unbalanced diet i.e. shortened villus height, enlarged crypt regions (Syme and Smith, 1982). It is unlikely in this case that the level of protease activity is sufficient for the animals needs and this result indicates that even though the protein level in the diet may be high in comparison to the wild animals diet some form of inhibition is effecting the final breakdown of peptides, or the diet is deficient in some essential amino acids or minerals. More enzyme protein is produced in the apical membrane to achieve the breakdown of peptides in the CRA compared with the WRA.

3.6 Conclusion

The results of this study show that apical membrane vesicles can be produced from abalone intestinal tissue with a purity that compares to other marine species and terrestrial mammals (refer chapter 2 2.10.1). Maltase, in this study, is the predominate carbohydrase found with sucrase showing reasonable activity in the WRA group. β-galactosidase activity is most likely non-specific galactosidase activity in the cytosol, however it may provide an indication of an increase in mucus secretory cells in the intestine. There is a difference in enzyme

activity generally between the CRA and the WRA groups. This may be a reflection of components of the protein and carbohydrate sources that is marine vs. terrestrial, and their interaction with the intestine. Antinutrient factors of these sources may be causing the differences seen, as a number of studies mentioned on fish have also observed similar properties to those seen here in this study. In particular, lectin binding, allergenic proteins and inhibitions due to mucus secretion seem to have the most influence. It is possible that minerals, in particular phosphorous, and amino acid deficiencies are seen. Alkaline phosphatase activity may provide an early indication of epithelial layer health in future studies in that when alkaline phosphatase levels are low then the appearance of the villus structure is more like that seen in the wild reared animals and if high then the tissue shows the adverse effects seen in the CRA abalone. A number of the properties seen with the enzyme analysis can be reflected in the observed morphological changes.

Further testing needs to be done to establish the effects of carbohydrate source and complexity, protein source and phosphorous availability for use in membrane and cellular metabolism functions. The effects of antinutrient factors found in terrestrial plants should also be studied, as potential limitations to abalone intestinal structure and function have been seen in this study.

Chapter 4

Effect of Diet Constituents on the Abalone *Haliotis laevegata* Intestinal Structure and Function.

4. Effect of Diet Constituents on the Abalone *Haliotis laevegata* Intestinal Structure and Function.

4.1 Abstract.

Feed is the largest operating cost in intensive aquaculture. Soyflour and fishmeal are the main protein sources in commercial diets in Australia. Cheaper alternatives such as legumes are under study. Known antinutrient factors (ANFs) of terrestrial plants may impact on the abalone intestinal structure and function thereby making them inappropriate feed sources for abalone.

The structure and function of abalone intestine was investigated by histological and biochemical analysis. Abalone were fed isonitrogenous diets using four legumes and soyflour as protein sources that were used raw or subjected to treatments of raw plus phytase and heat applied to the legume seeds and the soyflour.

The typical morphology of the intestinal mucosa of the abalone fed the legume diets were:

- Villi with simple folds,
- The columnar cell nuclei are more centrally located
- On the simple villus structure, indentations can be seen with cells generally non-vacuolated in this area,
- Granulated cells are often seen,
- A profound infiltration of haemocytes in the lamina propria and interspersed in the columnar cells,
- Crypt regions show numerous secretory cells.

Biochemical analysis showed that treatment of legumes and soyflour produced changes to the enzyme profiles; however, the changes seen are not necessarily similar with each protein source or treatment.

The morphology of the abalone intestine, in all cases, displayed characteristic features of an inflammatory response. The biochemical analysis of the intestine suggests inhibition of enzyme activity that may be caused by ANFs or components of the protein and carbohydrate in the diet and may be treatable by enzyme addition to diets or heating of the protein source.

4.2 Introduction:

In intensive aquaculture feed is the largest operating cost. Protein is essential for soft tissue growth and is the most expensive component in artificial diets. Fishmeal is used extensively in many aquaculture diets as it supplies protein and energy as well as providing essential fatty acids. (Lovell, 1992). In 1998 World fishmeal production was around 5 million tonnes, representing a reduction of 27% over the previous three years and fish oil production was down 40% to 0.7 million tones (Pike and Barlow, 1999). The use of fishmeal and fish oil in aquaculture is expected to continue to the year 2010 and is predicted to take around 45% of the fishmeal and 70% of the fish oil production on a global basis, representing an increase of 39% for each over present use (Pike and Barlow, 1999). Supply of fishmeal and fish oil for aquaculture diets may become unpredictable in future. Soybean meal is considered to have potential as a replacement for fishmeal in aquaculture feeds as it has an amino acid profile similar to fishmeal and its protein is highly digestible (Evans, 1992). Currently, in Australia, the main protein sources used in commercial diets are fishmeal and soyflour (Vanderpeer *et al.*, 1999). Only small amounts of fishmeal and soyflour are produced in Australia, the remainder being imported at large cost. In keeping with overall goals of reducing feed costs to the abalone industry, researchers are currently looking at locally grown legumes as cheaper alternatives to soyflour and fishmeal (Vanderpeer *et al.*, 1999). Legumes being locally grown would also provide a more predictable supply.

Legumes have been used in stock feeds in Australia, and in other countries, and have been the subject of many studies in both terrestrial and aquatic animals. Anti-nutrient factors (ANFs) associated with the legumes such as trypsin inhibitors, protease inhibitors, alkaloids, lectins and tannins are the main problem in using these products (Francis *et al.*, 2001). Some of the effects are seen on the intestinal structure as well as interfering with enzyme function. Another ANF present in legumes is phytate and has been linked with phosphorous deficiency due to it being indigestible in this form (Reddy *et al.*, 1982). Phosphorous is used in nucleic acids, cellular energy releasing reactions, metabolism of carbohydrates, amino acids and lipids and in cell membranes. A reduction to that required for normal functions may see limited growth rates. Supplementation of phosphorous in an artificial diet for abalone, using soyflour as the protein source, showed an increase in growth (Coote *et al.*, 1996). Treating of raw ingredients by moist and dry heat-treat (cooking) and enzyme supplementation have been tested in both terrestrial and aquatic species to ameliorate the effects of ANFs (Brenes *et al.*, 1993; Irish and Balnave, 1993; Longstaff and McNab, 1987; Pfeffer *et al.*, 1995; Ressler *et al.*, 1968; Rubio *et al.*, 1990; Rumsey *et al.*, 1993). The effects/benefits appear to be species

specific (Huisman and van der Poel, 1988). In addition to the above effects of ANFs physiologic stimuli such as antigenic proteins and inflammatory mediators accelerate intestinal mucus secretions, forming highly cross-linked aggregates with the physical properties of a gel. This mucus layer can impede the diffusion of noxious agents however still allow diffusion of low molecular weight solutes (Lamont, 1992).

In a previous study (Chapter 3 section 3.6) on abalone taken from the wild and abalone reared on two commercially available feeds, an enzyme analysis showed several potential factors for study. Factors highlighted including carbohydrate and protein source and type, phosphorous availability and effects of antinutrient factors (ANFs) on cellular function. In order to test a number of possibilities and provide another perspective on the use of legumes, four legumes were selected based on their inherent chemical composition and nutritive value. The four legumes were the yellow lupin (*Lupinus luteus*), field pea (*Pisum sativum* cv. Alma), vetch (*Vicia sativa* cv. Blanchefleur) and faba bean (*Vicia faba* cv. Fjord). In order to be able to compare results obtained in the previous commercial diets of chapter 3 a soyflour diet was also formulated.

In brief, these legumes were chosen for the following properties,

- Soyflour and lupins have little starch, high protein content and high insoluble non-starch polysaccharides (NSPs). In diet formulations this allows for the addition of an enzyme (phytase) for release of phosphorous and heat treatments to ameliorate anti-nutrient factors (ANFs).
- Field peas, faba beans and vetch have different endogenous starch ratio (amylopectin: amylose) and content, phytic acid and varying NSPs at different levels, which allows enzyme supplementation and heat treatments in diet formulation.

In this study there were three objectives:

1. To determine if available phosphorous is limiting and if so affects abalone intestinal morphology and enzyme function when using terrestrial plants in diets for abalone. Four legumes, lupins, peas, vetch and beans were compared with soyflour. Treatments of raw and raw plus an enzyme (phytase, a microbial enzyme) were made into isonitrogenous diets in order to achieve this.
2. To determine if heat treatment of the legumes prior to inclusion in diets overcomes anti-nutrient factors problems/effects often seen in other marine species intestines.

3. To determine if β-galactosidase activity can be used as a predictor of high mucus production as potentially indicated in the experiments of chapter 3. Two diets from the above will be selected based on the higher β-galactosidase specific activity in the apical membrane. The tissue of abalone fed these diets will have further examination to show their surface characteristics by scanning electron microscopy fitted with a cryo transfer system.

4.3 Materials and Methods:

4.3.1 Animals

The abalone used in this experiment were from stocks held at South Australian Research and Development Institute (SARDI) West Beach. The abalone stocks held at SARDI were originally obtained from a commercial hatchery. Thirty-six animals were used for each treatment group. Within each treatment group, nine of the abalone were used for histological studies and twenty-seven were used for biochemical analysis.

4.3.2 Diets and Feeding.

For detailed diet ingredient formulation see appendix A Table A1 and Table A2. Briefly fifteen isonitrogenous diets were used with legumes field peas (*Pisum sativum* cv Alma), lupins (*Lupinus luteus*), faba beans (*Vicia faba* cv Fjord) and vetch (*Vicia sativa* cv Blanchefleur) given three treatments, raw, raw plus enzyme (phytate), autoclaved (20 minutes, 121^{0}C, 140kPa). Defatted soyflour was used as the control with all three treatments applied. Thirty-six animals were used for each treatment group. Animals were fed to excess daily at 1700hrs for 14 days.

4.3.3 General Methods.

The general methods used in this study are detailed in Chapter 2 (General Materials and Methods), reference numbers are shown.

Aquarium System.	2.1
Abalone Tissue Sampling.	2.2
Histology.	2.3, 2.3.1, 2.3.2
Apical Membrane Preparation.	2.5
Electron Microscopy.	2.4.1, 2.4.2
Enzyme Assays.	
Alkaline Phosphatase	2.6.1
Maltase, sucrase and β-galactosidase	2.6.2

Leucine Amino-peptidase	2.6.3
Succinate dehydrogenase	2.7.1
Acid Phosphatase	2.7.2
Sodium/potassium ATPase	2.8
Protein.	2.9

4.3.4 Chemicals.

Glucose determination used a Boehringer-Mannheim test kit (Chromogen - ABTS) purchased from Adelab Adelaide, all other chemicals were the best available analytical grade and purchased from Sigma (Sydney Australia).

4.3.5 Statistical Analysis

The data are expressed as means ± SE. Significant differences between means were calculated with one-way analysis of variance on Genstat version 5 ($P<0.05$).

For simplicity in referencing abalone fed a particular protein source, the abalone intestine will be referred to after the protein source and or its treatment. For example the intestine of abalone fed the raw soyflour diet (S), raw soyflour + phytase (SP) or heated (autoclaved) soyflour (SH)

	Treatment		
Protein Source	Raw	Raw + Phytase	Heat
Soyflour	S	SP	SH
Lupin	L	LP	LH
Vetch	V	VP	VH
Pea	P	PP	PH
Bean	B	BP	BH

4.4 Results

Several notable observations were made during the dissection of the abalone in regard to the intestine. These were, the S and V groups were distended and full of fluid and 60% of the V group had little or no food particles in their intestine.

4.4.1 Morphological Studies:

The plates shown for the morphological studies are typical examples of the three (n=3) abalone examined for each legume and soyflour treatments. The intestinal sections studied are denoted section 1 and 2 as shown in Plates 2.1 and 2.2 p34.

Light Microscopy;

The morphology of the intestinal mucosa (section 1) (Plates 4.1-4.4) of the abalone fed the legume diets included:

- Villi have simple folds,
- The columnar cell nuclei are centrally located
- On the simple villus structure, indentations can be seen with cells generally non-vacuolated in this area,
- Granulated cells are often seen,
- A profound infiltration of haemocytes is present in the lamina propria and interspersed in the columnar cells,
- Crypt regions show numerous secretory cells.

Soyflour based diets (Plate 4.1 (a) to (f)):

Heavy folding is seen in the crypt regions of the S (Plate 4.1a) and SH (c) groups while the SP group (b) show no folding. The villus height is greatly reduced (approximately 60-70%) with the SH group (c). Indentations and folding are more frequent on the villus of the S group (a). Few goblet cells are seen in the S (a) and SP (b) groups and are more abundant in the SH group (c). Haemocytes are seen in all intestines of abalone fed soyflour-based diets. Nuclei were centrally located in the S (d) and SP (e) groups with the SP group showing a more even pattern while the SH group (f) show a random array.

Lupin based diets (Plate 4.2 (a) to (f)):

All intestine of abalone fed lupin based diets show heavy folding of the crypt regions. The villus height is reduced in the LH group (Plate 4.2 c). Indentations are more frequent on the villus of the VH group. There are few goblet cells seen in the LP group (b) with increasing numbers in the L (a) and LH (c) groups. The LH group (c) show numerous goblet cells on the villus proper and the crypt regions. Haemocytes are seen in all intestines of abalone fed lupin-based diets. The nuclei of the LP group (e) are located more towards the apical membrane than the L group (d) while the LH group (f) show a random array. Granulated cells are prominent on the villus of the LP group (e) as is the neutral mucin stains, which are prominent in the region where most granulated cells are located. The lamina propria of the LP (b) and LH (c) groups are much thicker than in the L group (a).

Vetch based diets (Plate 4.3 (a) to (f)):

The crypt regions of the V (Plate 4.3a) and VP (b) groups are heavily folded with most cells being secretory cells. The VH group (c) shows few goblet cells in comparison to the other treatments. Nuclei are centrally located in cells of the VH group (f) and in an even pattern whereas the V (d) and VP (e) groups are randomly placed. Haemocytes are reduced in number in VH (f). The intestine of all abalone fed all vetch treatments show a large number of granulated cells with fewer seen in the VH group (f).

Pea based diets (Plate 4.4 (a) to (f)):

The PP (b) and PH (c) groups have heavily folded crypt regions, an irregular nuclei pattern (e, f), a thicker lamina propria and more goblet and secretory cells than the P group (a). Very few goblet cells are seen in the P group (a). Villus height of the PP group (b) is greatly reduced in comparison to the P group (a). Haemocytes are seen in all intestines of abalone fed pea-based diets

Bean based diets (Plate 4.5 (a) to (f)):

Unlike other treatments of the legumes the intestine of abalone fed beans showed little difference with phytase treatment. Granulated cells are prominent in the intestine of abalone fed all bean treatments, as is the number of indentations in the villus and crypt regions. The BH group crypt regions are heavily folded, the lamina propria is thicker, villus height is reduced and there are more goblet and secretory cells than with other treatments. Haemocytes are seen in all intestines of abalone fed bean-based diets

The morphology of the intestinal mucosa (section 2), of the abalone fed the legume diets, generally included:

- The columnar cell nuclei are centrally located,
- A profound infiltration of haemocytes in the lamina propria and interspersed in the columnar cells,
- Granulated cells are often seen,
- On the mucosa, indentations can be seen with cells generally non-vacuolated.

Ultra-Structure Studies (SEM):

Surface Characteristics;

Plate 4.6 shows the epithelial surface of the typhlosole at the top, the middle section and the crypt region in the S (a, b, c) and V (d, e, f) groups. The selection of the S and V groups for this study was due to them having a high β-galactosidase activity (figure 4.2c). In the intestine of abalone fed these diets no underlying structures can be seen.

The top of the villus (Plate 4.6 a, d) show many layers of mucus made up of fine strands that form together to give a sponge-like appearance. In the V group (d), the strands are more numerous and produce a more dense sponge-like appearance. The middle section of the typhosole (Plate 4.6 b, d) has a distinct, vertical planned flat, dense ribbon or sheet-like appearance with a very fine and sparse covering of mucus over this area. The V group (e) has numerous thick strands that cover the ribbon shaped mucus. In the crypt regions (Plate 4.6 c, f), the mucus covering is thicker. The V group (f) has a distinct sheet with little exposure possible to the epithelial surface. The S group has various width strands, with the wider strands forming into small sheets, however the spaces between these strands would allow some exposure to the epithelial surface.

Epithelium Apical Region (Plate 4.7 (a) to (f)):

While most diets show similar features, the most prominent being the spherules that cover the majority of the surface of the villus, there are a few diets that have different features and are shown on Plate 4.7.

Typical arrangements of the intestinal surface of abalone fed lupins with all treatments showed little evidence of spherules in section 1 (Plate 4.7a) whereas section 2 (d) is similar to that seen in other abalone fed the other legume diets. In section 1 (a) cilia can been seen surrounding the apical surface of each cell

The heavy coating of mucus has not been removed by processing the tissue in abalone fed raw vetch (b) such that few underlying structures are seen. The VH group (e) shows the typical underlying structures however there was a disruption of the spherule structure and a reduction in number of spherules.

The B group is shown in (c & f) (section 1). Of note is the varying size of the spherules and the elongated secretory cells protruding past the apical membrane of the epithelial cells. The secretory cells have an opening at the lumen end where emerging secretory globules are often seen.

Transmission Electron Microscopy (Plate 4.8)

The plates show the typical arrangement over most areas of the VH group where there is an abundance of secretory cells. These secretory cells are easily distinguished from the goblet cells by the size of the secretory globules, which are approximately twice the size.

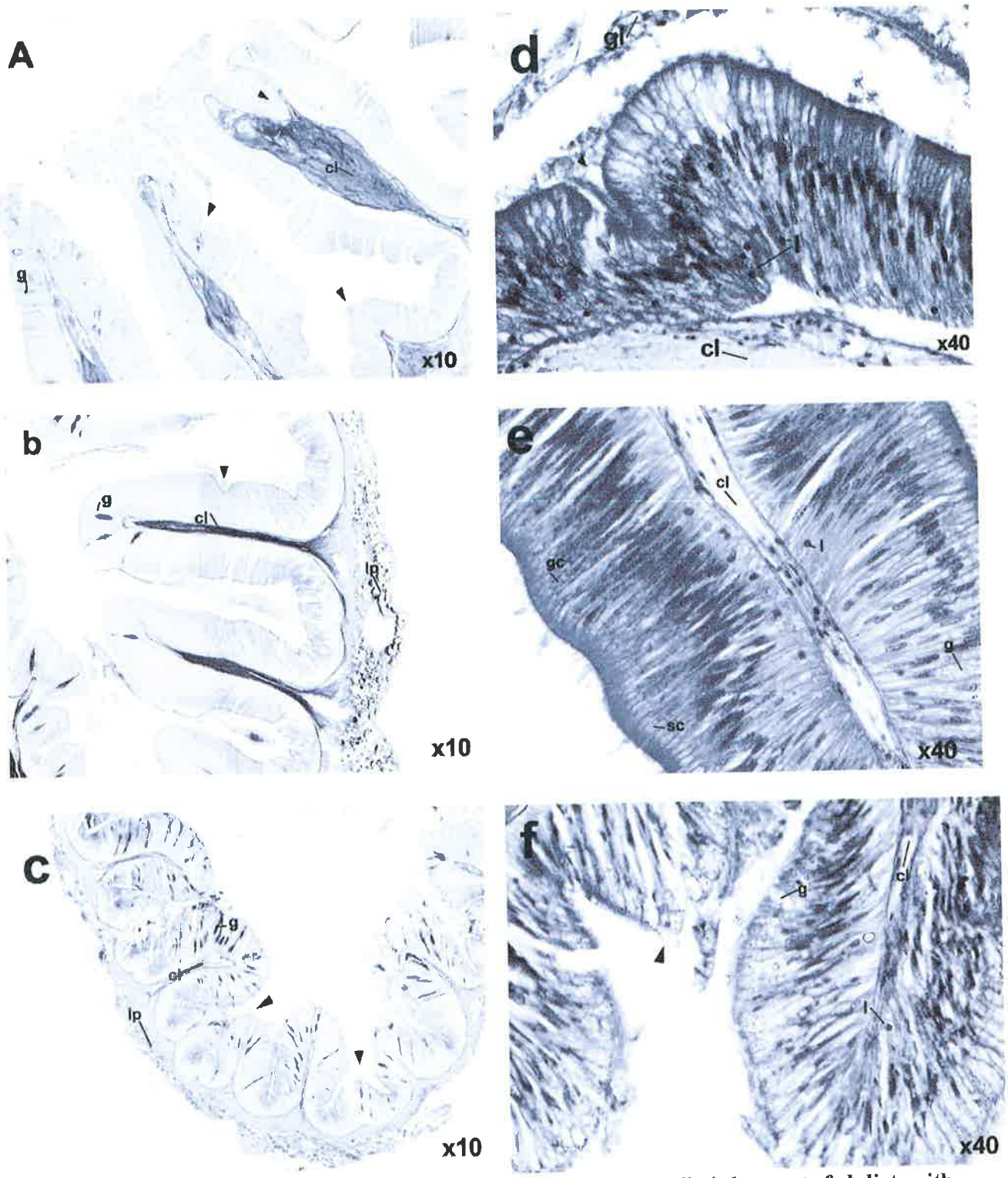


Plate 4.1: Detail of mucosa of intestine (section 1) of the abalone *Haliotis laevegata* fed diets with protein source (a, d) Soyflour, (b, e) Soyflour + phytase and (c, f) Autoclaved Soyflour. In all abalone villi with simple folds are seen. Indentations (arrowheads) along the villi and the crypt regions are more frequent and pronounced in the (a) diet than in (b). The crypt regions of (a) and (c) are heavily folded (arrowheads) with secretory cells abundant. Few goblet cells are seen in (a) and (b) and are more abundant in (c). The villus height of (c) is much reduced. Haemocytes are seen in all abalone. Nuclei are centrally located in (d) and (e) with no pattern seen in (f). Granulated cells are more prominent in (d) and (e). cl = central lacteal, g = goblet cell, gc = granulated cell, gl = glycocalyx, l = haemocyte, lp = lamina propria, sc = secretory cell.

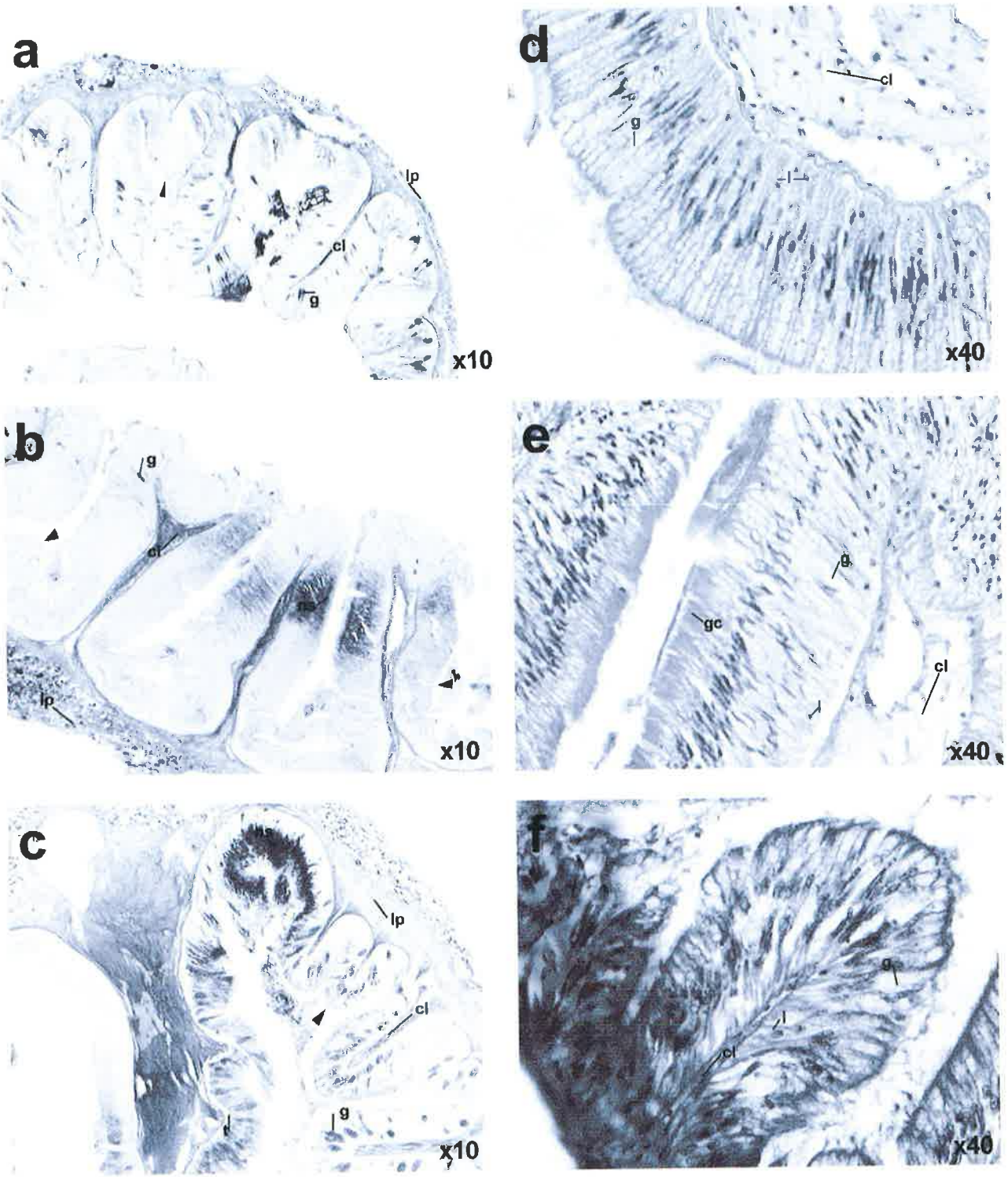


Plate 4.2: Detail of mucosa of intestine (section 1) of the abalone *Haliotis laevegata* fed diets with protein source (a, d) Lupins, (b, e) Lupins + phytase and (c, f) Autoclaved Lupins. In all abalone villi with simple folds are seen. The crypt regions of all abalone are heavily folded (arrowheads)with secretory cells abundant. Few goblet cells are seen in (b) with increasing numbers in (a) and (c). Indentations are more frequent on the villus of the VH group (c). The villus height of (c) is much reduced. Haemocytes are seen in all abalone. Nuclei are centrally located in (d) and (e) with no pattern seen in (f). A prominent neutral stain is seen in (c) in the crypt and typhlosole regions and in (b) on the upper regions of the villus. The lamina propria is thicker in (b) and (c). cl = central lacteal, g = goblet cell, gc = granulated cell, gl = glycocalyx, l = Haemocyte, lp = lamina propria, ns = nuetral stain, sc = secretory cell, t = typhlosole.

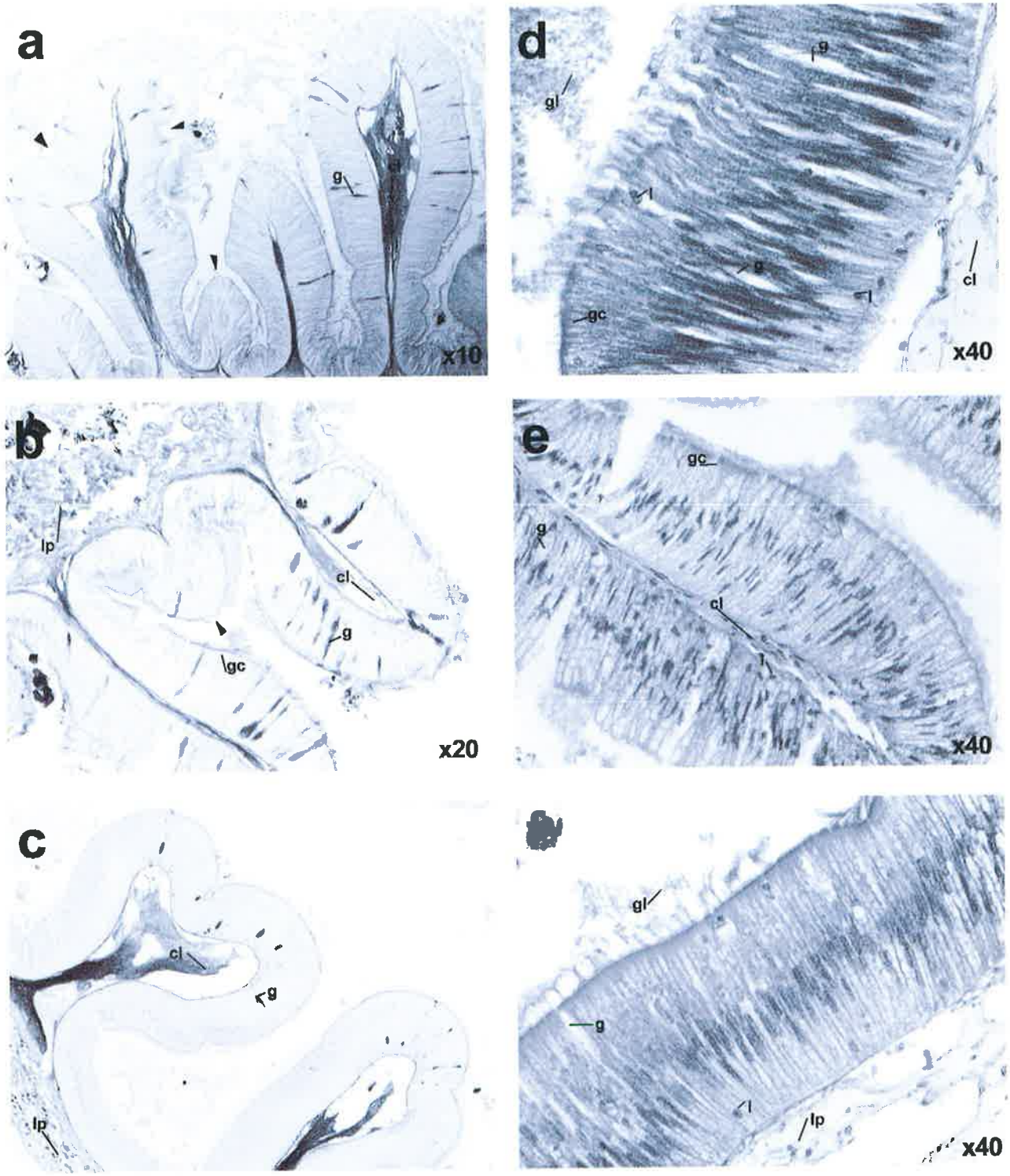


Plate 4.3: Detail of mucosa of intestine (section 1) of the abalone *Haliotis laevegata* fed diets with protein source (a, d) Vetch, (b, e) Vetch + phytase and (c, f) Autoclaved Vetch. In all abalone villi with simple folds are seen with indentations more prominent in (a). The crypt regions of (a) and (b) are heavily folded (arrowheads) with secretory cells abundant. Few goblet cells are seen in (c) with increasing numbers in (a) and (b). The villus height of all abalone is similar. Fewer Haemocytes are seen in (f) than in (d) and (e). Nuclei are centrally located in (f) with no pattern seen in (d) and (e). Granulated cells are more prominent in (d) and (e). cl = central lacteal, g = goblet cell, gc = granulated cell, gl = glycocalyx, l = Haemocyte, lp = lamina propria, sc = secretory cell.

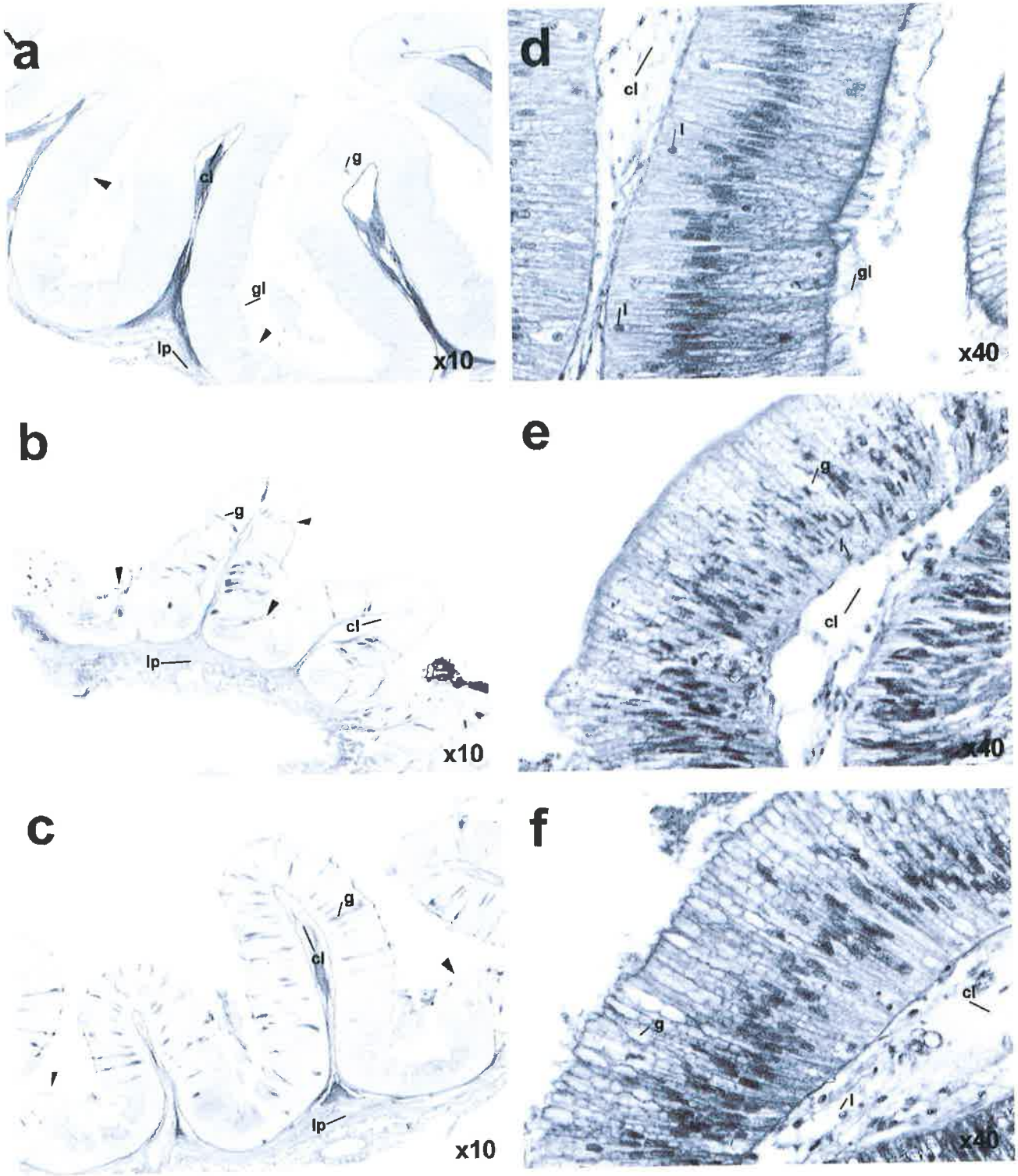


Plate 4.4: Detail of mucosa of intestine (section 1) of the abalone *Haliotis laevegata* fed diets with protein source (a, d) Peas, (b, e) Peas + phytase and (c, f) Autoclaved Peas. In all abalone villi with simple folds are seen with indentation on the villus in (b) (arrowheads). The crypt regions of (b) and (c) are heavily folded (arrowheads) with secretory cells abundant. Few goblet cells are seen in (a) with increasing numbers in (b) and (c). The villus height of (b) is much reduced. Haemocytes are seen in all abalone. Nuclei are basally/centrally located in (d) with no pattern seen in (e) and (f). The lamina propria is thicker in (b) and (c). cl = central lacteal, g = goblet cell, gc = granulated cell, gl = glycocalyx, l = Haemocyte, lp = lamina propria, sc = secretory cell.

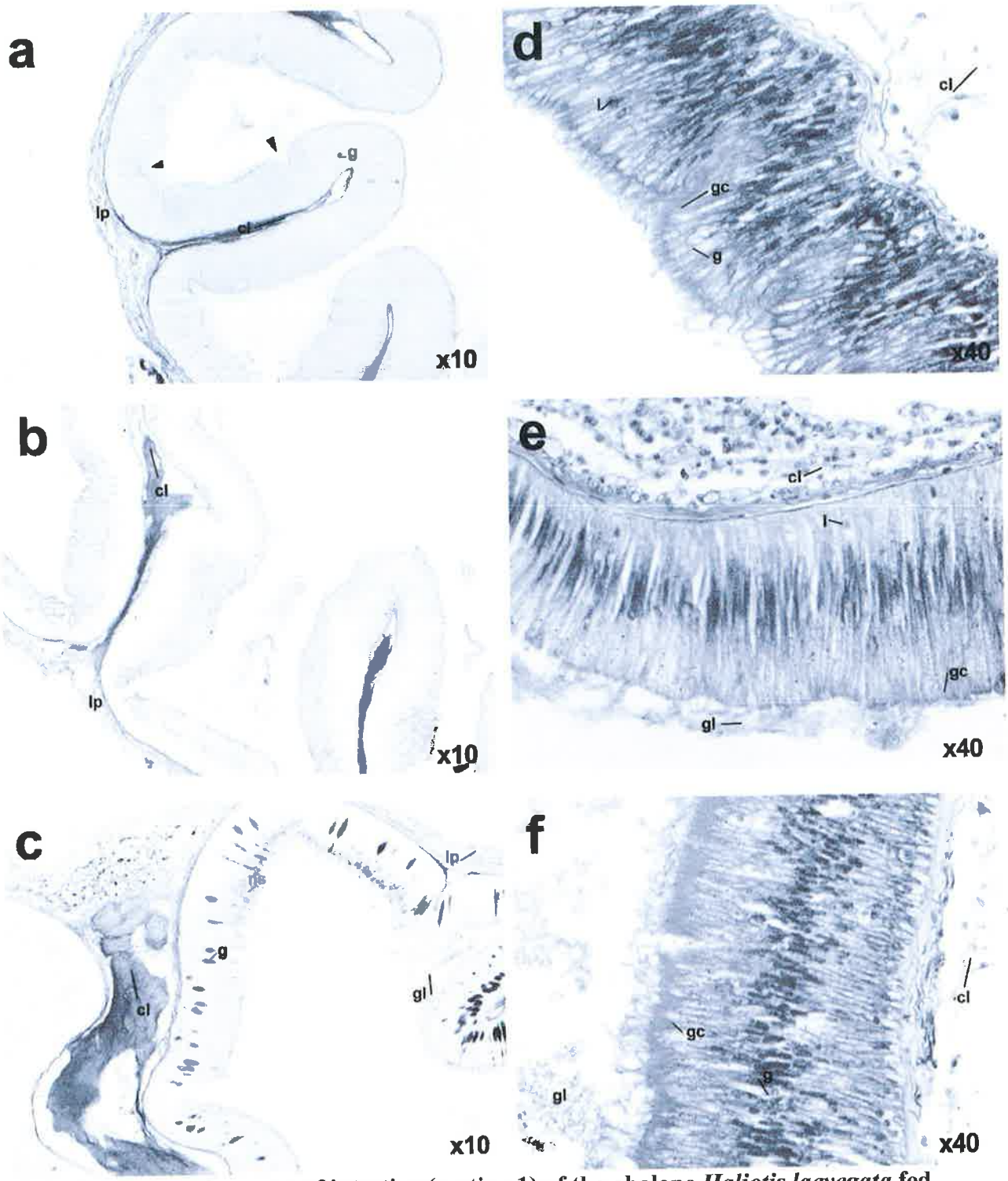


Plate 4.5: Detail of mucosa of intestine (section 1) of the abalone *Haliotis laevegata* fed diets with protein source (a, d) Beans, (b, e) Beans + phytase and (c, f) Autoclaved Beans. In all abalone villi with simple folds with indentation are seen (arrowheads). The crypt region of (c) is heavily folded (arrowheads) with secretory cells abundant. Few goblet cells are seen in (a) and (b) with increased numbers (c). The villus height of (c) is much reduced. Haemocytes are seen in all abalone. Nuclei are centrally located in all abalone. The lamina propria is thicker in (c). A neutral stain is seen in the crypt region of (c). Granulated cells are prominent in all abalone. cl = central lacteal, g = goblet cell, gc = granulated cell, gl = glycocalyx, l = Haemocyte, lp = lamina propria, ns = neutral stain, sc = secretory cell.

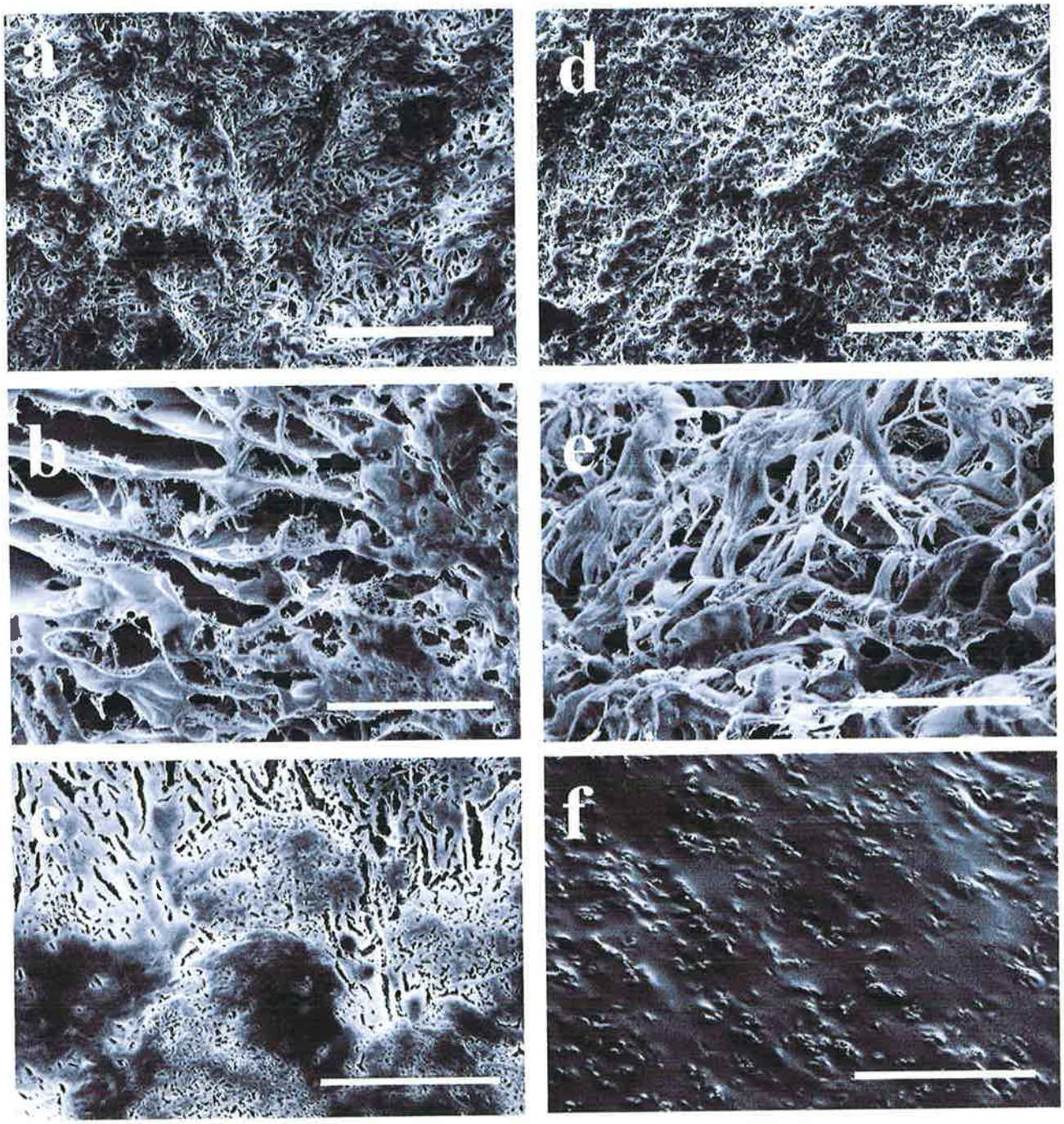


Plate 4.6: Epithelial surface of the abalone *Haliotis laevegata* intestine section 1 villus top (top of page), middle typhosole region (centre) and crypt region (bottom) showing surface mucus coating using SEM fitted with a cryo transfer system. Abalone fed soyflour is shown in plates a, b and c and abalone fed vetch plates d, e, f. Bar =10μm centre plates, Bar = 20μm top and bottom plates. Note in all areas with abalone fed vetch (d, e, f) there is a more dense coating of mucus with the crypt region a thick sheet with little exposure possible to the epithelial surface.

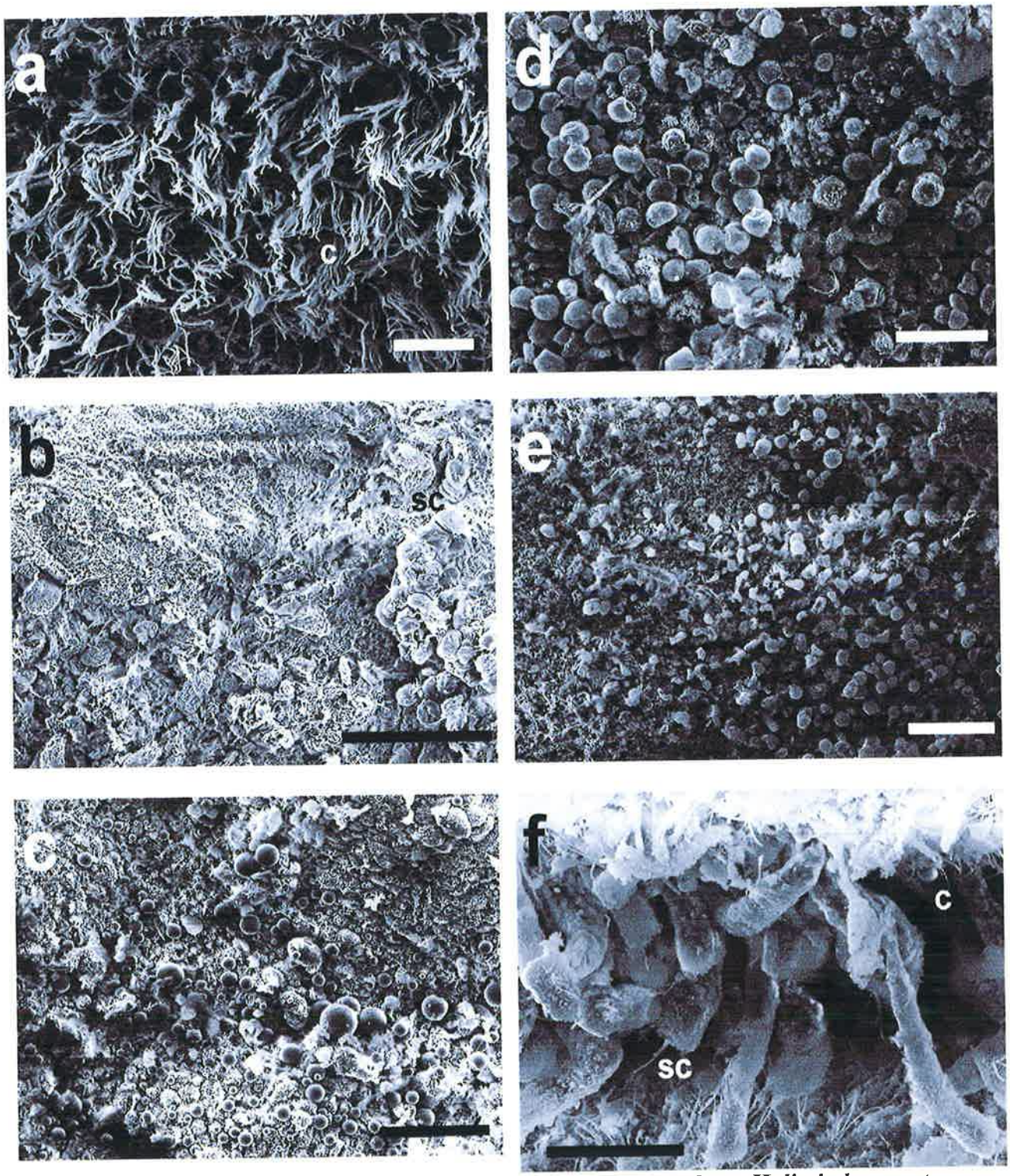


Plate 4.7: Epithelial surface of the intestinal villus of the abalone *Haliotis laevegata* using SEM. Typical arrangements of the epithelial surface are shown for abalone fed lupins in (a) section 1 and (d) section 2. Note that there is little evidence of spherules in section 1 whereas section 2 similar to that seen in other abalone fed legume diets. Abalone fed vetch (b) and autoclaved vetch (e) are shown (section 1), note the heavy coating of mucus that has not been removed by processing the tissue while underlying structures are seen in (e). Abalone fed beans are shown in (c and f)(section 1), note varying sizes of the spherules in (c) and secretory cells protruding past the epithelial apical surface in (f). Bar = 50μm (c, b, e), 10μm (a) and 20μm (d, f). (c = cilia, ec = epithelial cell, s = spherules, sc = secretory cells)

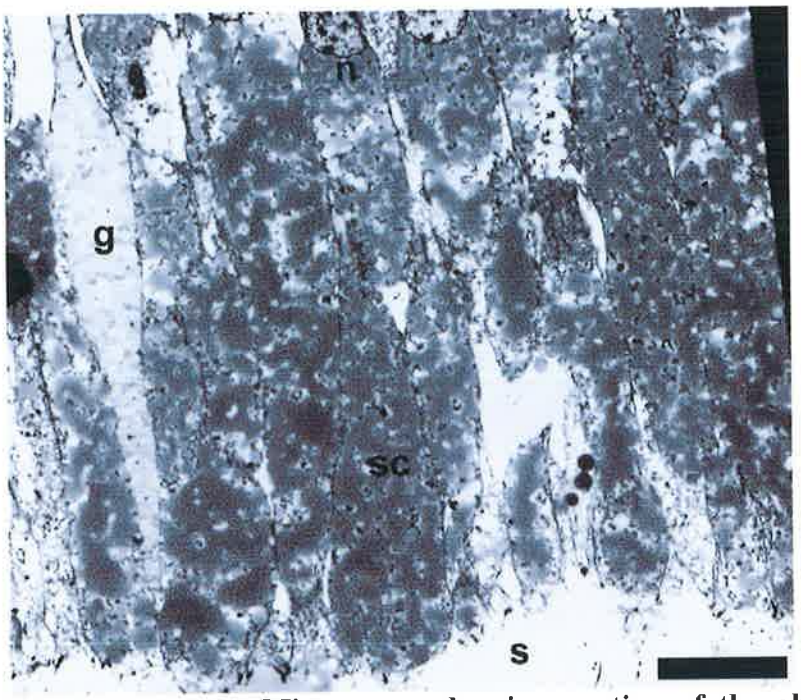


Plate 4.8: Transmission Electron Microscopy showing portion of the abalone *Haliotis laevegata* intestinal mucosa section 1. Abalone fed vetch is shown. Bar = 10μm. The plate is typical of most areas of the villus and shows the abundance of secretory cells in comparison to goblet cells and other cells. (g = goblet cell, n = nucleus, s = spherule, sc = secretory cell).

4.4.2 Functional Capabilities:

Apical Membrane Isolation

The apical membrane isolation procedure had been successful, yielding a suitable preparation for study of the effects of dietary components on enzymes. Protein recovery for the apical membrane fraction was between 7.5 and 12.2% of the protein present in the original homogenate.

4.4.2.1 Raw and Raw + Phytase Treatments:

With the addition of phytase enzyme to the bean diet, the intestine of abalone fed this diet showed no changes to any enzyme tested including the marker enzymes for cellular membranes other than the apical membrane and as such will not be discussed.

Alkaline Phosphatase

Alkaline phosphatase specific and total activity (figure 4.1) was significantly reduced in the LP and VP groups while the SP group had a significant reduction in specific activity only. The P group showed a significant increase in both specific and total activity.

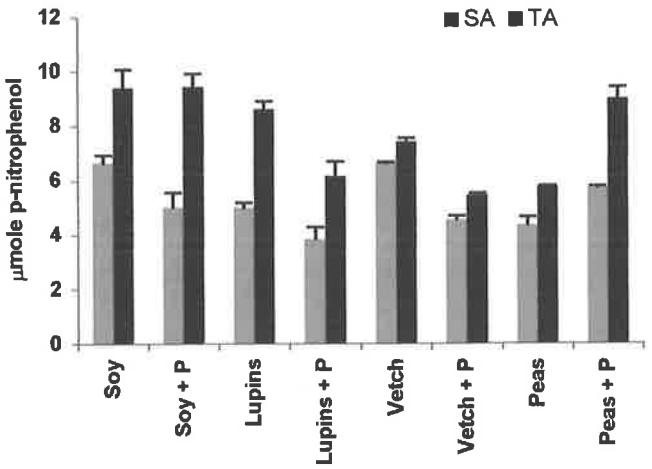


Figure 4.1: Effect of Dietary Protein Source with Treatments Raw and Raw + Phytase on Alkaline Phosphatase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity is expressed as µmole p-nitrophenol/mg protein/hr^{-1}, total activity is expressed as µmole p-nitrophenol/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. The TA of soy and soy+P were not significantly different at $P<0.05$. All SA and TA (within legume) of Lupins, vetch and peas were significantly different at $P<0.05$.

Carbohydrases.

Maltase was the predominant carbohydrase. β-galactosidase had greater activity than sucrase in all groups except for the intestine of abalone fed the lupin treatments (figure 4.2). Maltase activity in the VP group significantly increases in comparison to V group. The LP and SP groups show no change in specific activity while the PP group shows a significant decrease in both specific and total activities. There is a reciprocal change in sucrase and β-galactosidase activities with the abalone fed soyflour and vetch treatments. Sucrase increases while β-galactosidase activity decreases. The abalone fed pea treatments showed the opposite. The LP group shows a significant decrease in sucrase and β-galactosidase activity in comparison to the L group.

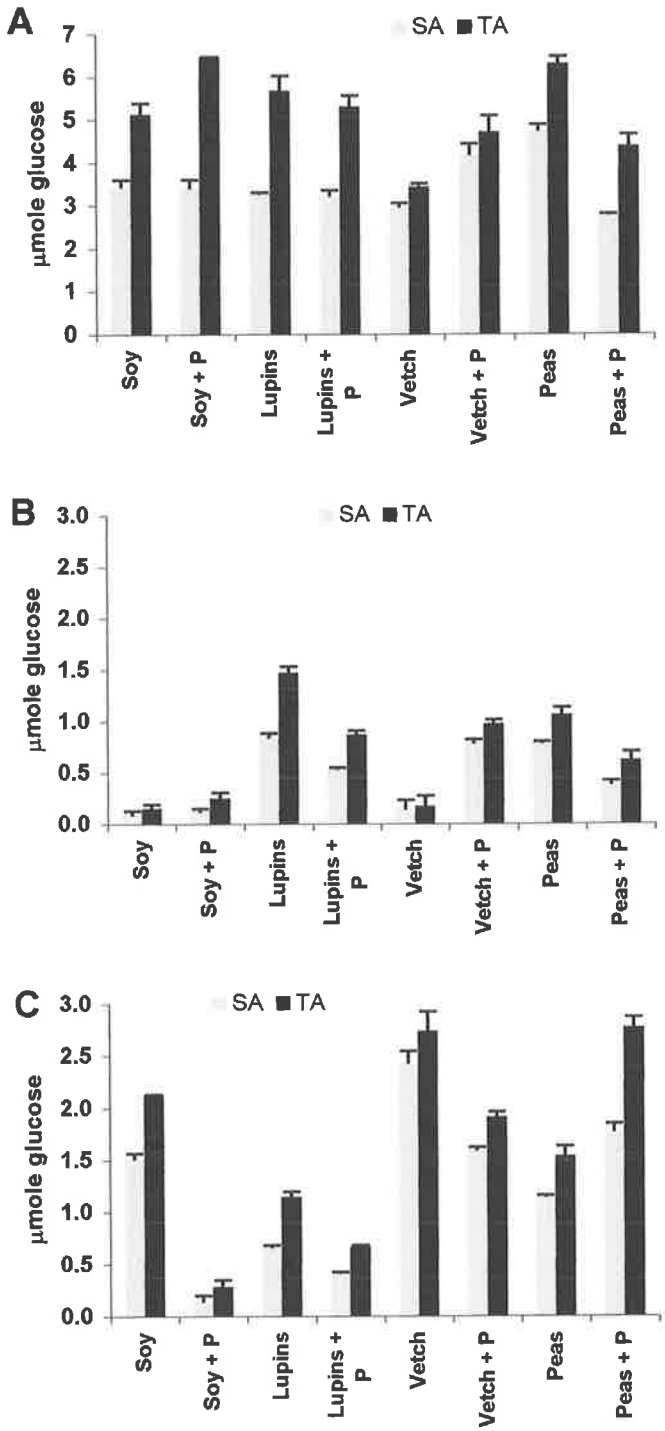


Figure 4.2: Effect of Dietary Protein Source with Treatments Raw and Raw + Phytase on Intestinal Carbohydrases Maltase (A), Sucrase (B) and β-galactosidase (C) Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Single factor ANOVA was used to assess significant differences for diet treatment. For maltase the SA of Soy, Soy+P, Lupins and Lupins+P were not significantly different (within legume) at $P<0.05$. For Maltase the TA of Lupins and Lupins+P were not significantly different at $P<0.05$.

Protease.

Leucine amino peptidase (figure 4.3) in abalone intestine fed all raw and raw plus phytase treatments show no significant change to the specific activity of the enzyme however, the total activity significantly increases in the SP and PP groups, was not changed in the VP group and is significantly reduced in the LP group.

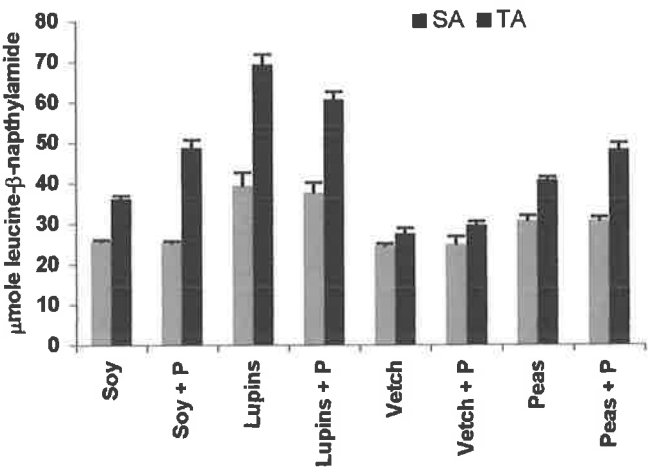


Figure 4.3: Effect of Dietary Protein Source with Treatments Raw and Raw + Phytase on Leucine Amino Peptidase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Single factor ANOVA was used to assess significant differences for diet treatment. Within Legume the SA of each treatment were not significantly different at $P<0.05$. The TA of Vetch and Vetch+P were not significantly different at $P<0.05$.

4.4.2.2 Legumes with Heat Treatment:

Alkaline Phosphatase.

All abalone intestine follow a similar pattern for alkaline phosphatase (Figure 4.4) activity when fed heat treated (autoclaved) legumes, which is, a significantly greater activity in respect to the raw treatment with the exception of the SH group, where heat treatment has decreased the specific activity only.

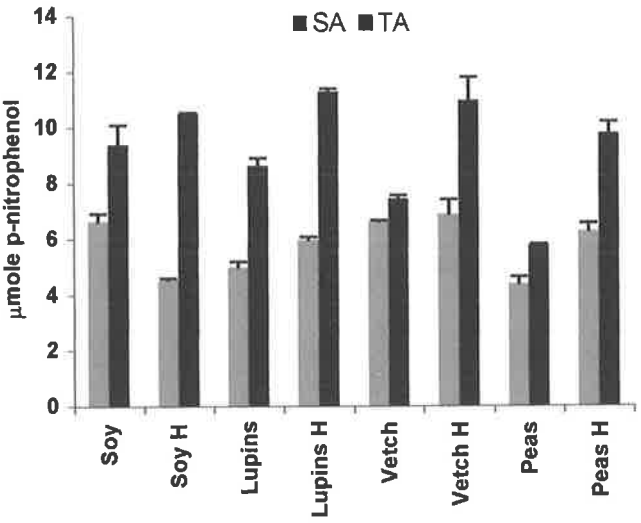


Figure 4.4: Effect of Dietary Protein source with Treatments of Raw and Heat on Alkaline Phosphatase Specific and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity is expressed as µmole p-nitrophenol/mg protein/hr^{-1}, total activity is expressed as µmole p-nitrophenol/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. All SA and TA (within legume) were significantly different at $P<0.05$

Carbohydrases.

Maltase is the predominant carbohydrase with β-galactosidase next highest except for the intestine of abalone fed lupin treatments where sucrase has the next highest activity. Maltase activity (Figure 4.5) with the SH group significantly decreases, the LH group remains the same as the L group while the PH and VH groups significantly increase. Sucrase activity significantly decreases in the LP and PP groups and the SP and the VP group significantly increases. β-galactosidase decreases in all abalone intestine fed diets with a heat treatment. The V and S groups (Figure 4.6 c) show the highest β-galactosidase specific activity and were 32% and 112% respectively greater than the next highest, the P group. Scanning electron microscopy was used to further test the V and S groups in their natural hydrated state for mucus coverage of the villus. Results are shown in Plate 4.6.

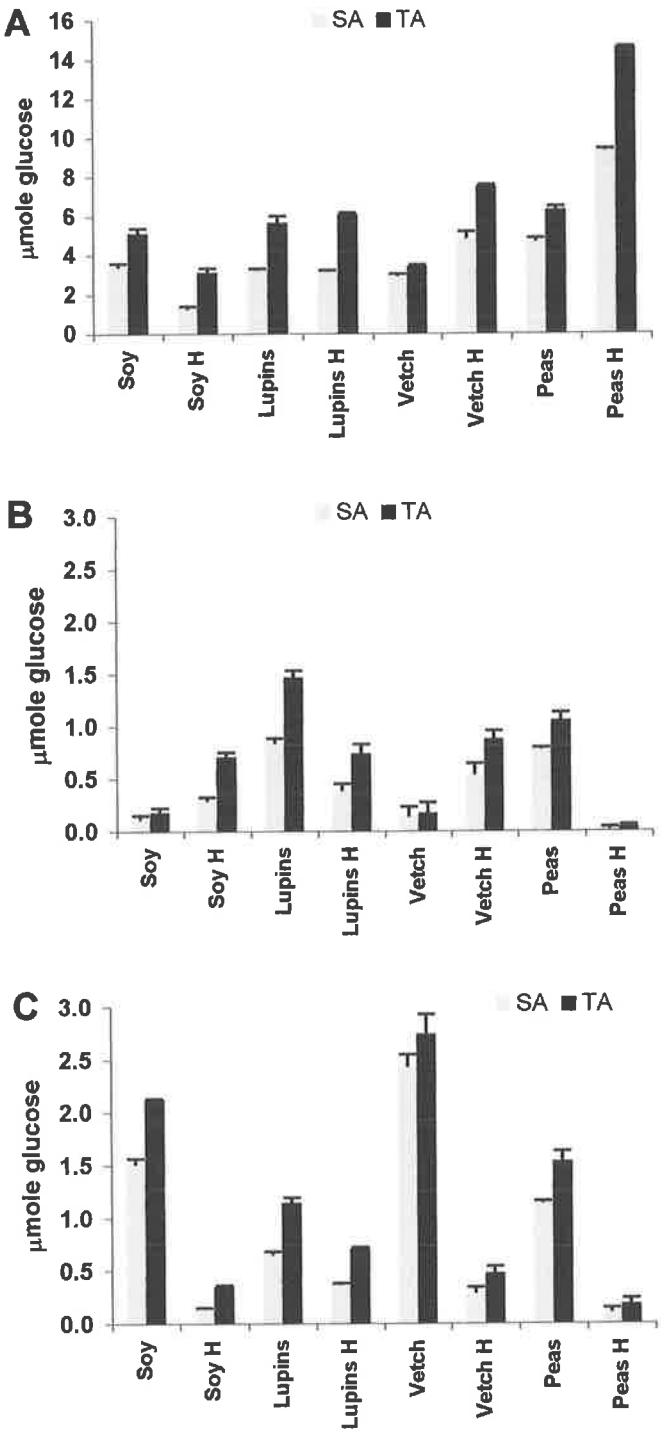


Figure 4.5: Effect of Dietary Protein Source with Treatments Raw and Heat on Intestinal Carbohydrases Maltase (A), Sucrase (B) and β-galactosidase (C) Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Single factor ANOVA was used to assess significant differences for diet treatment. For maltase SA and TA of Lupins and Lupins+H were not significantly different at $P<0.05$. Within Legume all SA and TA of other treatments were significantly different at $P<0.05$.

Protease.

Leucine amino peptidase (Figure 4.6) specific and total activities were significantly reduced in the abalone intestine fed all heat-treated legumes in comparison to the raw treatment.

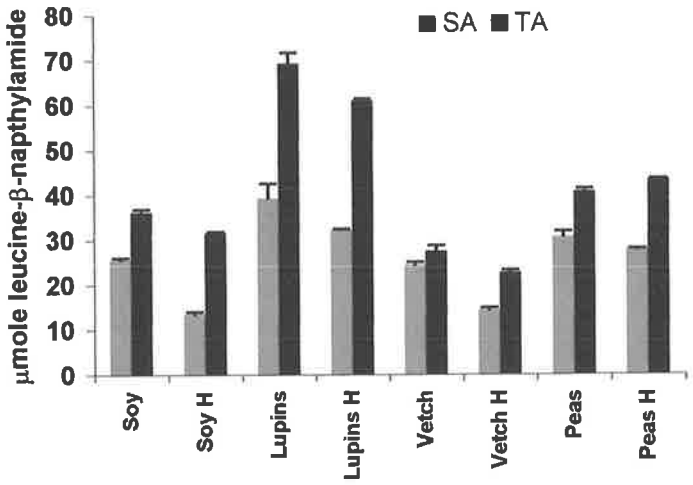


Figure 4.6: Effect of Dietary Protein Source with Treatments Raw and Heat on Leucine Amino Peptidase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume all SA and TA were significantly different at $P<0.05$.

4.5 Discussion:

Impact of Protein Source and Processing:

4.5.1 Raw Protein Source;

4.5.1.1 Morphological Studies,

The morphological changes seen in the intestine of abalone fed diets based on soyflour and the legumes lupins and vetch shows the same responses to that seen in the previous study (Chapter 3 section 3.5.1) with the commercial reared abalone. All show the characteristic features of inflammation, which are an infiltration of haemocytes, granulated cell increase in the lower villus regions and increased goblet and other secretory cells especially in the crypt region (Baeverfjord and Krogdahl, 1996; Ciancio and Chang, 1992; Crawford, 1994). The intestine of the abalone fed beans shows a similar response with the exception that the crypt region which is not enlarged or folded however when study of the tissue is done by scanning electron microscope (SEM) (Plate 4.7f) secretory cells are seen protruding past the epithelial

surface both in the crypt region and on the lower region of the villus. Soyflour and legumes when included in animal diets have shown to cause adverse effects on the morphology of the intestine. The changes have been ascribed to the presence of antinutrient factors (ANFs) particularly lectins, protease inhibitors, allergenic proteins (Huisman and van der Poel, 1988; Liener, 1994), the cyanoalanine compounds common in vetch (Ressler *et al.*, 1968) and tannins in beans (Marquardt and Ward, 1979). The differences seen here in this study may reflect the particular ANF composition of the seeds. The abundance of secretory cells in the V group (Plate 4.8) is clearly seen and is likely to be a response to the ANFs in vetch. The morphology of the L group in section 1 (Plate 4.7a) of the intestine has few spherules and while it is still not known what function the spherules provide, if they do play some part in the final digestion and uptake of food, i.e. a substitute for microvillus seen in mammalian and marine fish (Buddington and Diamond, 1987; Smith and Peacock, 1989), then a section of the intestine without this structure would have to compromise the efficiency of the animal in normal maintenance function. If this were the case then the abalone would have to rely on the second section of the intestine to digest and uptake nutrients. Further testing on the individual sections of the intestine may provide the answer to this question. The B group (Plate 4.7c) has varying sizes to the spherules in section 1 and the V group also show a disruption to the spherule structure (Plate 4.7e) in section 2 of the intestine. If the spherules have a microvillus like function then digestion and uptake of nutrients may be compromised in these regions. In a previous study on the pyloric ceca of tuna, a disrupted microvillus related to decreased digestive enzyme function and amino acid uptake (Kemp, 1997). The P group morphology has similar features to the wild reared abalone seen in the previous study (Chapter 3) and as peas are considered to be relatively free of ANFs (Petterson and MacIntosh, 1994) it gives credence to the suggestion that the morphological differences are due to the ANF composition of the other legumes.

4.5.1.2 Functional analysis:

The intestine of abalone fed all treatments of the bean diets show very little difference in enzyme profiles suggesting that where improvements have been seen in the performance of other animal species with treatment of beans (Bhargava and O'Neil, 1979; Marquardt and Ward, 1979), these improvements are not reflected in abalone. As such the functional analysis has not been shown and will not be discussed.

Alkaline Phosphatase,

The intestine of the S and V groups were distended and full of fluid, which is typical of both ruminants and monogastrics with low food intake or feed restriction. Deprivation of feed has been shown to increase alkaline phosphatase (AP) activity (Bamba *et al.*, 1990; Majumbar and Panda, 1989) and here in this study the S and V groups have the highest AP levels, this change may be related to feed intake. During dissection of the intestine from the V group, 60% of the abalone had little or no food particles in their intestine. Vetch was included at higher levels than soyflour in the diets and may not be palatable for abalone due to particular components such as the high content of cyanoalanine compounds (Ressler *et al.*, 1968). The immune-like response seen in the S, L and V groups can be an indicator of stress in the abalone and may have increased AP activity. Stress is another factor that has been shown to increase AP activity (Mizuno *et al.*, 1992). In terrestrial plants available phosphorous can be limited due to the phosphorous being bound in phytic acid (Liener, 1994). There may be an indication of this bound phosphate in the S, L and V groups. A function of AP is to dephosphorylate molecules liberating inorganic phosphate which in this case may be necessary due to limited available phosphorous and may be the reason for the high AP levels seen here (Mizuno *et al.*, 1992). The P group shows the lowest level of AP in comparison to the other raw legume treatments and it is interesting to note that the histology of the intestines of abalone fed this diet shows well-defined villus and crypt structure and intracellular features such as nuclei placed more towards the basal area of the cell and vacuolated cells suggesting mature cells and hence more functional cells. The AP specific activity and total activity was similar which suggests little inhibition by ANFs or other factors to the AP enzyme in the intestine of abalone fed this diet.

Carbohydrases.

The diets formulated for this study were isonitrogenous and due to the varying levels of protein in soyflour and each legume the inclusion levels of pregelled waxy maize starch as well as the endogenous starch level varied with diet. A summary is shown below.

	% Inclusion in Diet	% Endogenous Starch in diet	%Pregelled Starch in Diet
Soyflour	33	Trace	43
Lupins	39	Trace	37
Vetch	64	21	13
Peas	74	33	2

It is difficult to compare the true effects of the legumes on carbohydrase activity (Figure 4.2) in the abalone intestine in this trial as the inclusion levels of pre-gelled waxy maize starch

(99%amylopectin) vary considerably. Vanderpeer *et al.*, (1999) found the pre-gelled waxy maize starch to be highly digestible (92.5%) in the abalone. As pregelled starch is very similar in structure to the natural red algae (Craigie, 1974) consumed by abalone, it would be expected that glucose, the final product of hydrolysis of starch, may provide the stimulation for maltase activity (Guyton and Hall, 1996). The soyflour and lupin diets have the highest inclusion level of pre-gelled maize starch and as such it would be expected that high levels of maltase would be seen. This is however not the case as both S and L groups shows moderate level of maltase activity in comparison to the abalone fed the other raw legume sources. This high level of pregelled starch may in fact be limiting the maltase activity. High inclusion levels (38-41%) of pregelled starch has been shown to reduce digestibility and growth in other marine species (Hemre *et al.*, 1979; Henrichfreise and Pfeffer, 1992; Pfeffer *et al.*, 1995). The V group also shows moderate maltase activity however in this diet the level of pregelled starch is much lower than the soyflour and lupin diets and it may be that the effects of ANFs in vetch are responsible for the maltase levels. This also applies to the S and L groups as in all cases there appears to be an inhibition of the specific activity as the specific activity and total activity was considerably different. The increase in the amount of enzyme may be to compensate for the decreased specific activity. The raw pea diet effectively contains only endogenous starch of which 60% is amylopectin the remainder amylose (Booth *et al.*, 2001). The maltase and sucrase activities were higher in the P group and may be a result of the starch type present in peas, in that the abalone has to use more digestive enzyme for the final breakdown of this endogenous starch. The SA and TA of peas were similar suggesting less inhibition of enzyme function and may be indicative of the lower ANFs seen in peas.

Protease.

Protease activity was particularly high in abalone fed the lupins based diet in comparison to those fed the other diets however there is a considerable difference between the SA and the TA in the lupin based diet groups. The S, V and P groups protease activity is low which may indicate that the amount of enzyme present in the tissue is sufficient for the abalone needs. In the L group it is possible that some form of inhibition of the protease enzyme is seen as the SA is much lower than the TA.

4.5.2 Raw Protein Source Plus Phytase:

4.5.2.1 Morphological Studies,

The morphology of the SP, LP and VP groups show a slightly more even villus structure however there is still heavy folding of the crypt regions of the LP and VP groups. This may

be attributable to increased available phosphorous however other factors are still influencing the morphology of the intestine. With the appearance of improvement in the mucosal structure and the more mature cell features such as the nuclei placement and more vacuolated cells seen phosphorous may be more available for cellular metabolism and hence a flow on effect to an improved final digestion and uptake of nutrients may be implied. The PP group morphology shows the opposite effect in that the intestinal structure resembles one where some factor/s are having a profound effect. The epithelial layer resembles that of an immature intestine as noted above and we would expect the functional capacity of this intestine to be reduced.

4.5.2.2 Functional Analysis.

Alkaline Phosphatase.

Phytic acid in plants can be responsible for a decreased availability of phosphorous in diets however this may be overcome by using a phytase to hydrolyse the phytic acid into inositol and phosphates or phosphoric acid (Reddy *et al.*, 1982). Phytate also binds the minerals zinc, iron and calcium and it has been shown that alkaline phosphatase is dependant on zinc for activation in abalone (Lan *et al.*, 1995; Tan *et al.*, 2001), and other species (Mizuno *et al.*, 1992). The addition of phytase enzyme shows a reduction in alkaline phosphatase activity in SP, LP and VP groups, which suggests that micronutrient supply is not a factor. However, the release of phosphorous by this enzyme would mean there is fewer requirements for the dephosphorylation of phosphorous by alkaline phosphatase therefore less requirement for large amounts of alkaline phosphatase protein in the apical membrane. High levels of phosphate, pyrophosphate, adenosine triphosphate (ATP) and glucose-phosphate compounds inhibit alkaline phosphatase activity (Pappas, 1982), and may also be the likely reason for the decrease in this case. This result may be significant in that phosphorous is used in cellular membranes, ATP, AMP, DNA, RNA and metabolism of amino acid, lipids and carbohydrates and animals fed on these diets would have greater growth and health status. Coote *et al.*, (1996) found that addition of 0.5% NaH_2PO_4 to a soyflour-based diet significantly increased (7.9%) growth rates. The Japanese abalone *Haliotis discus hannai* requires an available dietary phosphorous at 1.15% inclusion in a diet for optimal growth (Tan *et al.*, 2001). The digestibility of cystine increases significantly with peas treated with phytase (Vanderpeer *et al.*, 1999). Cystine is known to inhibit alkaline phosphatase activity (Mizuno *et al.*, 1992) and in the PP group, which shows an increase in alkaline phosphatase, the abalone may be increasing the total protein in the apical membrane to counter the inhibition.

Carbohydrases.

Phytase has altered the levels of carbohydrates but in different ways however when a comparison is made with the raw ingredients the VP group stands out for the changes in maltase and sucrase activity. The specific activity of maltase in the intestines of VP is much higher than V and may be due to the altered membrane structure of the cells in that the enzymes are more stable in the apical membrane and can work more efficiently. The metabolic processes of the cell may have also increased allowing more enzymes to be synthesised. Following this argument the opposite would apply to the intestine of the PP group where maltase activity was reduced. There is an interesting pattern emerging with the SP, VP and PP groups with sucrase and β-galactosidase activity, in that, if a decrease in sucrase is seen β-galactosidase increases and vice versa. The SP and VP groups show an improvement in the morphology of the intestine while the PP group shows deterioration and this may be reflected in the sucrase and β-galactosidase activity. If we look at the SP and VP results, the question is, does a sucrase increase and a β-galactosidase decrease with a concurrent structural improvement relate to a better diet? With the PP group does a sucrase decrease and a β-galactosidase increase with concurrent deterioration in morphology relate to a poorer diet? In many studies a deterioration in morphology of the intestine has related to functional deficiencies and growth impairment (Baeverfjord and Krogdahl, 1996; Dunsford *et al.*, 1989; Francis *et al.*, 2001; Hisayasa *et al.*, 1992; Li *et al.*, 1991; Pusztai, 1989).

Protease.

The addition of phytase to the legumes has made no major differences to the activities of leucine amino peptidase, which may be expected as the phytase enzyme is used to release phosphorous from phytic acid.

4.5.3 Heat Treated Protein Source.

4.5.3.1 Morphological Studies.

The intestinal structure of all groups except the VH group is typical of a protein deficient diet, i.e. shortening of the villus height, thickening of the crypt region and an increase in the crypt region. The protein deficiency is most likely due to a maillard reaction caused by the high heat applied to the seeds. The maillard reaction effects lysine in particular, due to the non-digestible amino-sugar complex formed, and may cause this amino acid to be limiting (Hurrell and Carpenter, 1974). Vandepeer *et al.*, (1999) found in these diets lysine digestibility was significantly decreased. In contrast to this the VH group shows a marked improvement in structure, (nuclei are centrally located and in an even pattern, haemocytes are

reduced in number, fewer granulated cells) suggesting a more mature intestine. With the VP group there was a slight improvement in the structure of the villus however it was considered that ANFs were also affecting the intestine. Heat treatment is often used to ameliorate the effects of ANFs and in this case it appears that this is so. Heat treating vetch has also reduced the mucus covering in the VH group as is as expected as the number of secretory cells is much reduced in this diet.

4.5.3.2 Functional Analysis.

Alkaline Phosphatase Activity.

Heat treatment or cooking of legumes causes the formation of insoluble salts rendering phosphate in limited supply (Reedy *et al.*, 1982). The formation of insoluble salts may be responsible for the increase in alkaline phosphatase seen here in this study due to the lower availability of phosphorous. In all abalone the increase in TA in the intestinal epithelial cells apical membrane, in comparison to the raw groups, suggests an increase in alkaline phosphatase enzyme and may be to compensate for the low phosphorous availability.

Carbohydrases.

The effect of the heat treatment on carbohydrates can be seen with the VH and PH. The bulk of the starch in these diets is from the seed itself and the moist heat treatment would have pregelled the starch making it more available for amylase digestion and hence become more readily digested. The released glucose would increase the synthesis of the maltase enzyme. Maltase activity in abalone intestine has increased with both these diets. Autoclaving soyflour and lupin seed, which have little starch content, had no effect on the starch. The starch in content of these diets is the waxy maize pre-gelled starch, which has not been included in the heat treatment. Abalone fed on soyflour and lupin diets show a reduced total intestinal carbohydrase activity and would be related to the marked changes in intestinal structure as well as decreased phosphorous availability for cell metabolic functions. The VH group shows improved morphology of the intestine, an increase in sucrase activity and a decrease in β-galactosidase activity suggesting a better diet.

Proteases.

Autoclaving the legumes at 121°C has probably caused a maillard reaction and/or cross-linking of proteins rendering the proteins indigestible by blocking sites of enzyme attack (Maynard *et al.*, 1979). In all cases the protease activity is reduced, more so in SH and VH.

Vandepeer *et al.* (1999) using the same diets as this current study found a decrease in the availability of lysine in all these diets with the greater decreases in SH and LH.

4.5.4 Mucus Cover:

β-galactosidase was not enriched in the apical membrane and is contrary to that seen in mammalian and some marine species (Boge *et al.*, 1993; Pelleter *et al.*, 2001; Shirazi-Beechey *et al.*, 1990). The S and V groups showed the greatest specific activity and intestinal tissue was taken from these abalone and examined by scanning electron microscopy in the tissue's natural state. The mucus coating of the intestine was dense in both cases with the V group being more so. Galactosides are abundant in the intestine and present in mucosal cells and secreta (Allen, 1981). The β-galactosidase activity seen here may have been more influenced by these galactosidases than those in the feed itself. As such this enzyme may be used as an indicator of a mucus coating with high levels seen as a thick coverage. The crypt regions of intestinal tissue taken from these diets show a heavy folding with secretory cells in abundance. Transmission electron microscopy of the V (Plate 4.8) shows more secretory cells than vacuolated or granular cells, which shows that mucus is being produced in abundance rather than cells producing normal digestive functions. There are ramifications to energy use by the abalone in that mucus production has been found to account for 23-29% of the energy lost (Peck *et al.*, 1987). An increase as seen here in the intestine would mean the amount of excess energy that would normally be converted to growth or stored as glycogen in the foot may be reduced, thereby effecting abalone production and the quality of the meat product. In effect a repartitioning of the energy would be seen away from storage reserves to the normal functions required for maintenance of the abalone. A thick coating of mucus as seen here may reduce the amount of feedstuff that would be in close contact to the epithelial cell surface for final digestive breakdown. This would then have a flow on effect on the amount of nutrient available for transport into the cell and once again effect reduced meat quality and growth. With a change in the mucus environment it may also allow enteropathogens to adhere to the mucus affecting the health of an animal (Lamont, 1992).

4.6 Conclusion:

This study indicates that using terrestrial plants in abalone diets can have a detrimental effect on the structure and function of the abalone intestine. In their raw state the legumes lupins, vetch and beans as well as soyflour all showed morphology of an inflammatory response to some ingredient in the diets most likely due to the wide variety of antinutritional substances known to exist in each of these protein sources. The use of peas in diets had less effect on the

abalone intestinal structure and function probably because of the limited amount of ANFs found in this protein source.

Alternatively ANFs that restrict the bioavailability of essential nutrients may account for the observed morphological and biochemical changes. The reduction in phytate and subsequent liberation of phosphorous and other micronutrients may also account for the observed effects. With the addition of phytase to a diet the improvement seen varies in degree between these diets however the structural alterations imply that when sufficient phosphorous is available we see a more regular and possibly functional intestine.

Destruction of heat labile ANFs of terrestrial plants may also be important as this treatmentm may increase protein digestion and provide better use of available energy for growth and maintenance. Autoclaving the feed at the temperature used in this study was too high and it would be necessary to heat protein sources at lower temperatures and produce diets for testing to obtain optimal benefit from heat treatment.

The diets were designed to be isonitrogenous, which caused varying inclusion levels of pre-gelled starch. No clear effect of the legume source could be deduced in regard to carbohydrase activity. There is an indication that the reciprocal changes seen in sucrase and β-galactosidase activity may imply a better diet however further testing needs to be done on carbohydrate source and type. Inclusion levels also need to be made the same to see if any effect can be seen.

There is strong evidence from this study that β-galactosidase activity appears to be related to the amount of mucus secreted by the abalone in the intestine. Any dietary component that may induce a secretory response should be carefully looked at before inclusion is made in abalone diets.

Chapter 5

Carbohydrate Digestion in the Abalone *Haliotis laevegata* Intestine: Effects of Processed Maize Starch, Endogenous Legume Starch and Inclusion Level.

5. Carbohydrate Digestion in the Abalone *Haliotis laevegata* Intestine: Effects of Processed Maize Starch, Endogenous Legume Starch and Inclusion Level.

5.1 Abstract:

The importance of carbohydrate source, type and inclusion levels as possible sources of metabolizable energy in manufactured abalone diets are still under investigation.

The altered function of abalone intestinal carbohydrases was investigated using isonitrogenous diets with various levels of pregelled waxy maize starch either singly or in combination with native sources of starch from the legumes, field peas, faba beans and vetch, by biochemical analysis. The effects of the inclusion level of the legume source in the diets were also included in the investigation.

The analysis showed that high (37-43%) inclusion levels of pregelled starch reduces carbohydrase activity in abalone intestine in comparison to levels of 10% and 23-25%. Greater ratios of amylopectin: amylose increases intestinal carbohydrase activity. A reduction in the inclusion level of the legumes field peas and vetch increase intestinal carbohydrase activity.

Abalone diets using native starches from terrestrial sources may benefit from amylopectin inclusion (10 –25%) (pregelled starch) in diets as intestinal carbohydrase activity increases at these levels. The differences seen in carbohydrase activity cannot be solely isolated to inclusion levels, starch type or kaolin addition as all may have some effect individually or in combination. Faba beans and vetch require more study to as to the effects of inherent anti nutrient factors before consideration is made for their use as protein and carbohydrate sources in abalone diets. Field peas may be used providing another source of protein is included allowing a reduction in their inclusion levels.

5.2 Introduction:

Dietary carbohydrate serves largely as a source of digestible energy in animal production (e.g. poultry, pig, sheep and cattle). In manufactured abalone diets, while the digestible energy level is important, equally important is that starch is used to bind the manufactured 'pellet' to stop it from dissolving in water in a short time period.

Digestibility of native starches from legumes by the abalone (*Haliotis laevegata*) is highly variable with digestibility coefficients of 45% for vetch, 49% for field peas and 65% faba beans (Vanderpeer *et al.*, 1999). The range of variation increases with processed starches with digestibility coefficients ranging from low levels of 34% in semolina, to high levels such as 93% in pregelled waxy maize starch (Vanderpeer, 2001). The chemical composition of native starches in legumes varies especially with the ratio of amylose to amylopectin (Petterson and MacIntosh, 1994). Amylose is considered more a long term storage of glucose as it is not as easily hydrolysed as amylopectin which allows rapid hydrolysation by enzymes due to the many non-reducing ends in its structure (Mathews and van Holde, 1990). Hydrothermic treatment (pregelling) alters both the starch composition and how water-soluble the starch becomes, both providing easier access to non-reducing ends by enzymes (Mercier and Feillet, 1975). The growth performance of the aquatic species, trout, salmon and cod have shown considerable improvements when pregelled starch has been included in manufactured diets (Arndt *et al.*, 1999; Hemre *et al.*, 1979; Henrichfreise and Pfeffer, 1992). Greater growth rates have been attributed to a rapid hydrolysis of gelatinised starch by α-amylase, however, at high inclusion levels the digestibility of pregelled starch and the animals performance is reduced (Bergot and Breque, 1983; Pfeffer *et al.*, 1991). In a previous study (Chapter 4 section 4.6) using legume-based diets, starch source, type and also treatments were highlighted as possible factors providing different effects on the abalone intestinal carbohydrase activity.

The legumes selected in the previous study had the following important properties that were utilised in this study:

- Soyflour and lupins have little starch. In diet formulations this allows for substitution of a preferred starch.
- Field peas, faba beans and vetch have different endogenous starch ratio (amylopectin: amylose) and content, which allows for starch ratio changes by supplementation of preferred starches.

The preferred starch used for this experiment was pregelled waxy maize starch as this has 99% amylopectin content similar to the red algae normally consumed by wild abalone *Haliotis laevegata*. The results of this experiment using the hydro-thermic treatment of native starches from legumes may be important to companies who manufacture abalone diets by extrusion.

In this study there are three objectives:

1. To determine if pregelled starch inclusion levels affect the carbohydrase activities in the intestine of abalone. This was examined using diets formulated with soyflour, lupins and pregelled starch plus a reduced inclusion of starch by substitution with the inert filler, kaolin.
2. To determine if starch type affects the carbohydrase activities in the intestine of the abalone. Vetch, field peas and faba bean diets as formulated for the previous study (Chapter 4) was used with partial substitution of pregelled starch for endogenous starch.
3. To determine if reduced inclusion levels of the legume effect enzyme activities in the intestine of abalone. Diets as formulated for 2 were used. Additional diets with a reduced inclusion level of the above legume sources with a partial substitution of soyflour to maintain the diets isonitrogenous were used.

5.3 Methods:

5.3.1 Animals

The abalone used in this experiment were from stocks held at South Australian Research and Development Institute (SARDI) West Beach. The abalone stocks held at SARDI were originally obtained from a commercial hatchery. Thirty-six animals were used for each treatment group. Within each treatment group, nine of the abalone were used for histological studies and twenty-seven were used for biochemical analysis.

5.3.2 Diets and Feeding.

Soyflour, lupins, vetch, peas and beans diet formulations are shown in appendix A. Starch and the reduced inclusion level diets are shown in Appendix A Tables A1-A4 inclusive. Briefly the changes made to the diets above are shown below. Animals were fed to excess daily at 1700hrs for 14 days.

	Base Diet			Treatment Diet		
	% Endogenous Starch in Diet		% Pregelled in Diet	% Endogenous Starch in Diet		% Pregelled in Diet
	Amylopectin	Amylose	Pregelled	Amylopectin	Amylose	Pregelled
Soyflour	-	-	43	-	-	10
Lupins	-	-	37	-	-	10
Vetch	7	14	13	2.5	4.5	25
Peas	20	13	2	10	7	23
Beans	10	14	11	6	8	25

5.3.3 General Methods.

The general methods used in this study are detailed in Chapter 2 (General Materials and Methods), reference numbers are shown.

Aquarium System.	2.1
Abalone Tissue Sampling.	2.2
Apical Membrane Preparation.	2.5
Enzyme Assays.	
Alkaline Phosphatase	2.6.1
Maltase, sucrase and β-galactosidase	2.6.2
Leucine Amino-peptidase	2.6.3
Succinate dehydrogenase	2.7.1
Acid Phosphatase	2.7.2
Sodium/potassium ATPase	2.8
Protein.	2.9

5.3.4 Chemicals.

Glucose determination used a Boehringer-Mannheim test kit (Chromogen - ABTS) purchased from Adelab Adelaide, all other chemicals were the best available analytical grade and purchased from Sigma (Sydney Australia).

5.3.5 Statistical Analysis

The data are expressed as means ± SE. Significant differences between means were calculated with one-way analysis of variance on Genstat version 5 ($P<0.05$).

For simplicity in referencing abalone fed a particular protein source, the abalone intestine will be referred to after the protein source and or its treatment. For example the intestine of abalone fed the raw soyflour diet (S), the intestine of abalone fed on a diet containing soyflour or lupins with a kaolin substitution for portion of the pregelled starch (SR)(LR) or the intestine of abalone fed on a diet with a reduced inclusion level of vetch, maintained isonitrogenous by a substitution of soyflour, and an increased inclusion of pregelled starch (VRI).

Protein Source	Treatment: Raw	Reduced Pregelled Starch	Reduced Starch and Inclusion Level
Soyflour	S	SR	-
Lupin	L	LR	-
Starch	ST	STR	-
Vetch	V	-	VRI
Pea	P	-	PRI
Bean	B	-	BRI

5.4 Results:

Apical Membrane Isolation.

The apical membrane isolation procedure had been successful, yielding a suitable preparation for study of the effects of dietary components on enzymes. Protein recovery for the apical membrane fraction was between 7.0 and 11.6% of the protein present in the original homogenate.

5.4.1 Reduced Pregelled Starch Inclusion:

Alkaline Phosphatase.

The reduction in starch had no effect on alkaline phosphatase activity in the L and ST groups; however, there was a significant reduction in both specific and total activity with the SR group (Figure 5.1).

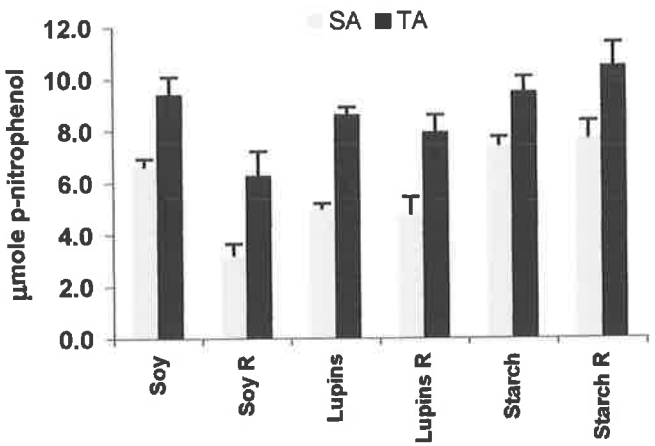


Figure 5.1: Effect of Diets with Reduced Starch on Intestinal Alkaline Phosphatase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity (SA) is expressed as µmole p-nitrophenol/mg protein/hr; total activity (TA) is expressed as µmole p-nitrophenol/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume the intestinal apical membrane SA and TA for the treatments lupins and lupins R and starch and starch R were not significantly different at $P<0.05$.

Carbohydrases.

Maltase is the predominant carbohydrase activity in the intestine of all abalone (Figure 5.2). There are no significant differences in the specific activity of maltase in all groups however the total activity increases significantly in the SR and STR groups while the LR group significantly decreases. Sucrase activity significantly increases in all groups. In all abalone intestine fed the reduced starch diets, the β-galactosidase specific and total activities decreases. There is a reciprocal change with sucrase and β-galactosidase activities in that sucrase activity increases in abalone intestine fed the reduced pregelled starch diets while β-galactosidase increases.

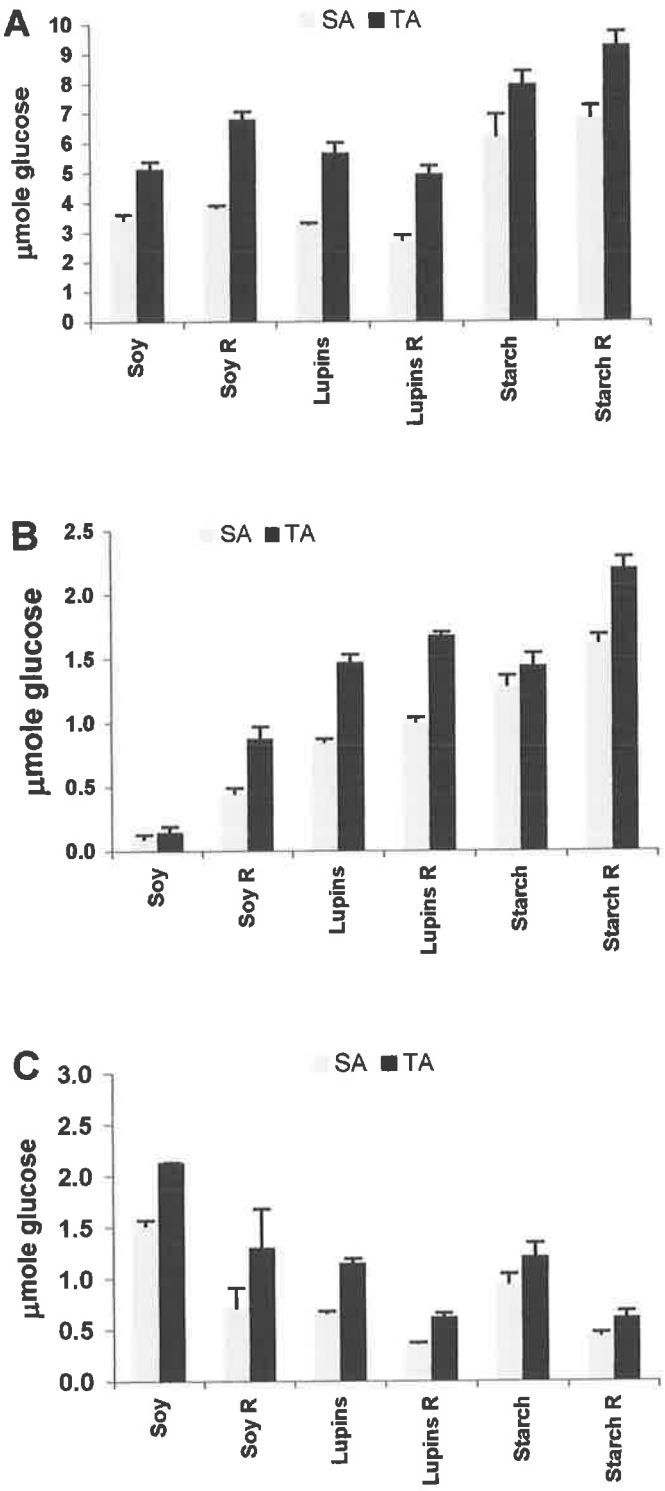


Figure 5.2: Effect of Diets with Reduced Starch on Intestinal Carbohydrases Maltase (A), Sucrase (B) and β-galactosidase (C) Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole glucose/mg protein/hr; total activity (TA) is expressed as µmole glucose/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within Legume the intestinal apical membrane maltase SA for soy, lupins and stach treatments were not significantly different at $P<0.05$.

Protease.

Leucine amino–peptidase (LAP) activity is significantly increased in the SR group in comparison to the S group in both specific and total activity (Figure 5.3). There are no differences seen in activities in the LR group while the SR group shows significantly increased specific activity but no difference in total activity.

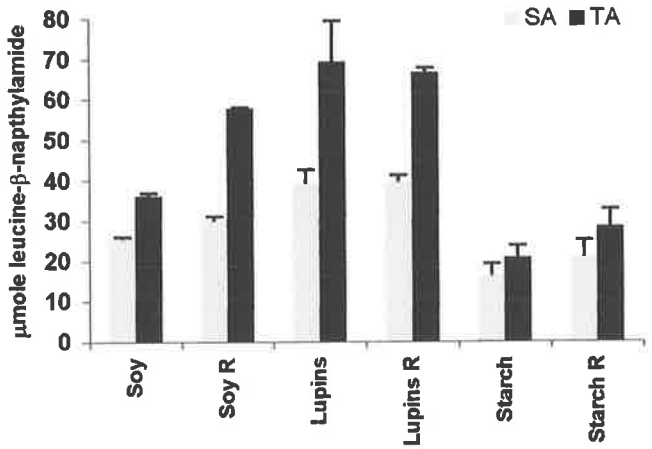


Figure 5.3: Effect of Diets with Reduced Starch on the Protease Leucine Amino-peptidase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole leucine-β-napthylamide/mg protein/hr, total activity (TA) is expressed as µmole leucine-β-napthylamide /gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume the intestinal apical membrane SA and TA for the lupin treatments were not significantly different at $P<0.05$. Within legume the TA for the starch treatments were not significantly different at $P<0.05$.

5.4.2 Reduced Legume Inclusion and Altered Starch:

Alkaline Phosphatase.

Alkaline phosphatase specific activity significantly increases in the PRI and BRI groups while no change is seen with the VRI group (Figure 5.4). Total activity significantly increases with the VRI and PRI groups while beans shows no change.

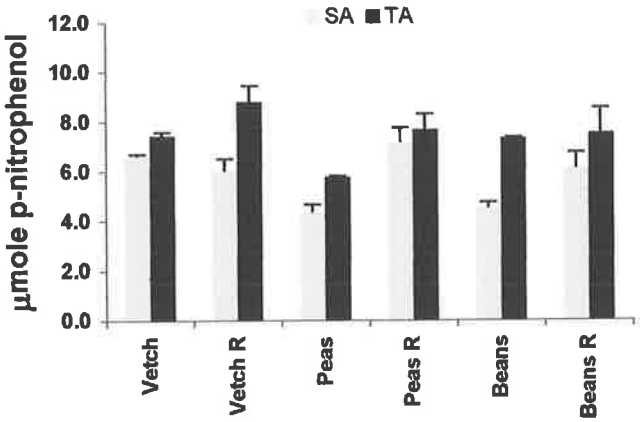


Figure 5.4: Effect of Diets with Reduced legume Inclusion and Altered Starch Type on Intestinal Alkaline Phosphatase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity (SA) is expressed as µmole p-nitrophenol/mg protein/hr; total activity (TA) is expressed as µmole p-nitrophenol/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume the intestinal apical membrane SA of the vetch treatments were not significantly different at $P<0.05$. Within legume the intestinal apical membrane TA for the beans treatments were not significantly different at $P<0.05$.

Carbohydrases.

There is no difference in maltase specific activity (Figure 5.5) in all groups however the total activity in the PRI and BRI groups significantly decreases while the VRI group significantly increases. Sucrase activity significantly increases in the VRI and PRI groups while the BRI group shows no change. β-galactosidase total activity significantly decreases in all groups as does the specific activities of VRI and PRI while beans remains the same. There is a reciprocal change in the sucrase and β-galactosidase activities in the VRI and PRI groups.

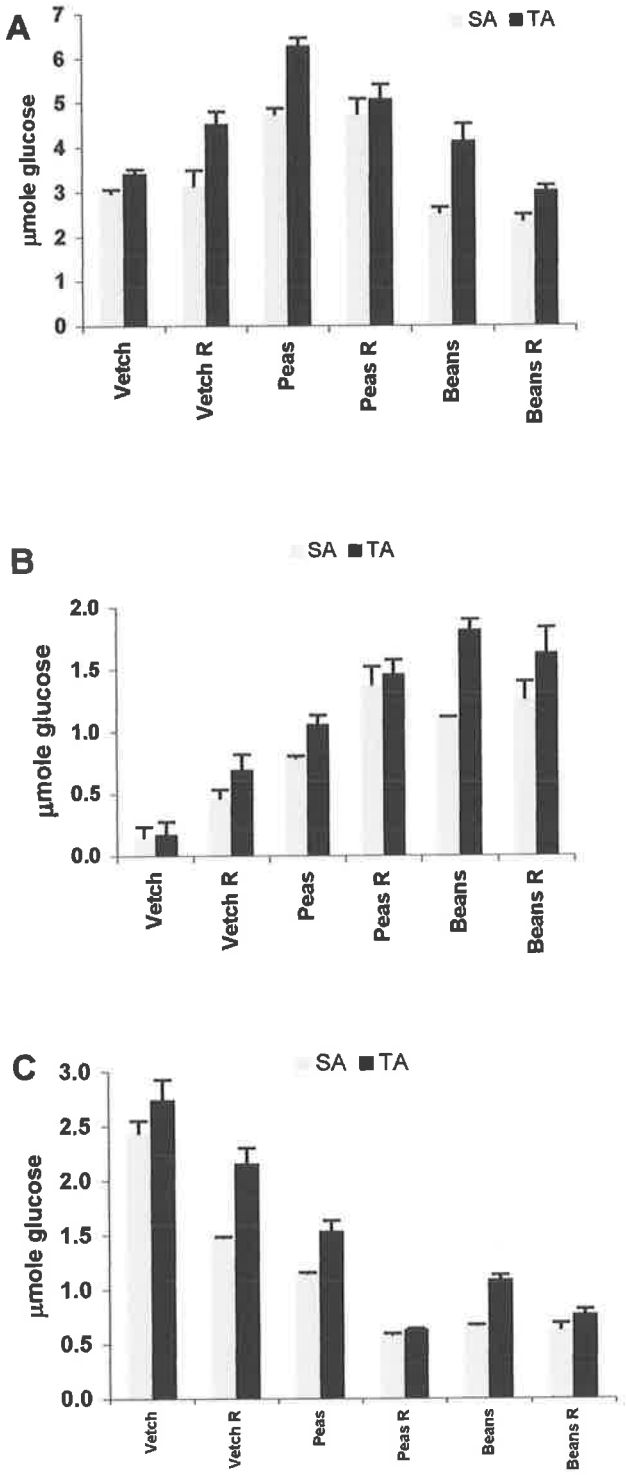


Figure 5.5: Effect of Diets with Reduced Legume Inclusion and Altered Starch Type on Intestinal Carbohydrases Maltase (A), Sucrase (B) and β-galactosidase (C) Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole glucose/mg protein/hr; total activity (TA) is expressed as µmole glucose/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume the maltase SA of vetch, peas and beans were not significantly different at $P<0.05$. Within legume the sucrase SA and TA of the beans treatments were not significantly different at $P<0.05$ as was the β-galactosidase Sa for the beans treatment.

Protease.

The effects of reduced legume inclusion level and altered starch levels on leucine amino-peptidase (LAP) are shown in figure 5.6. The PRI and BRI groups show significant increases in specific activity while the total activity remains the same. The VRI group shows a significant increase in specific and total activity.

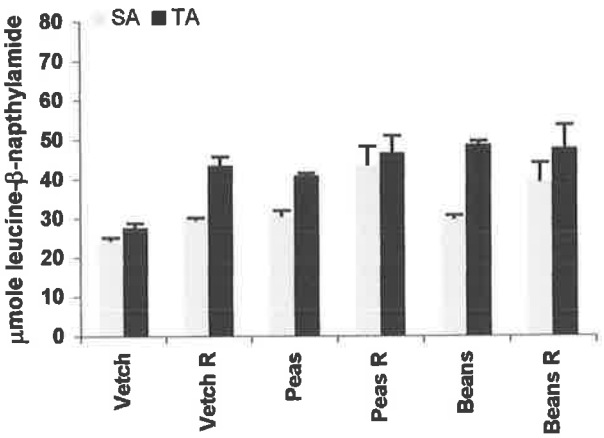


Figure 5.6: Effect of Diets with Reduced Legume Inclusion and Altered Starch Type on the Protease Leucine Amino-peptidase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole leucine-β-napthylamide/mg protein/hr, total activity (TA) is expressed as µmole leucine-β-napthylamide /gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume the intestinal apical membrane TA of the peas and beans treatments were not significantly different at $P<0.05$.

5.5 Discussion:

5.5.1 Reduced Pregelled Starch Inclusion:

Alkaline Phosphatase and Protease.

The experimental diets used in this study had been designed specifically to test the effects of starch and are by no means practical diets. The starch diet is 98% pregelled starch with no protein source in the diet. This diet would cause stress to the abalone and the effects can be seen in the alkaline phosphatase (AP) activities, which are the highest of the diets tested. The leucine amino-peptidase (LAP) activities for the ST and STR group are also low indicating that there is little need for protease activity in this diet. Britz *et al.*, (1996) found that protease and carbohydrase activity peaks between 12 and 18 hours after feeding returning to a base level by 24 hours. The LAP activity seen (intestine sample taken between 12 and 18 hours after feeding) in the ST and STR group may well be near a base level as there would be no stimulation from ingested protein to raise these levels. There is, however, a significant

increase in LAP specific activity in the STR group indicating some stimulation, which can only be attributed to the presence of kaolin. The SR group also shows a similar increase in specific activity and may also be related to kaolin.

The use of silicates, kaolin plus others, has been used in pig and poultry nutrition for many years with some researchers observing beneficial effects on growth, feed efficiency and nutrient utilization (Mumpton and Fishman, 1977). A major effect of inert fillers is to slow down the passage of nutrients in the digestive system (Dias *et al.*, 1998). A slower passage of nutrients allows more time for the enzymes to hydrolyse substrates further and hence lead to the increased efficiencies noted above. AP activity may be an example of this as the SR group shows a significant reduction in activity. A slower passage would allow greater release of phosphorous from digested feedstuffs with more available for metabolic processes and therefore less need to hydrolyse and release phosphorous by the AP enzyme. It is possible that a decrease in the effects of antinutrient factors (ANFs) in the soyflour caused by these ANFs binding with the kaolin and lessening interference with the enzyme function is occurring. The addition of naturally occurring clay materials to diets is thought to provide a buffering action by interacting with components of diets (Britton *et al.*, 1978; Collings *et al.*, 1980). It is interesting to note that the AP and LAP activities are not changed in the LR group. In this and the previous experiments the greatest LAP specific activity seen has been in the abalone fed the lupins and commercial diet 1 (40 μmole leucine-β-napthylamide/hr), which may well be the maximal activity of this enzyme. The slower passage of foodstuff through the gut may also push the peak enzyme activity to a later time. It would be highly unlikely that the differences seen above would be outside this peak period as the time of kill was at the peak times found by Britz *et al.* (1996), 12-18 hours after feed intake. The peak period has an 8-hour duration, to see a marked effect on the enzyme activity there would have to be a delay of at least 6 hours.

Carbohydrases.

In all these diets the starch was pregelled waxy maize starch (99% amylopectin) such that any differences seen with the carbohydrase would be attributable to the levels of starch in the diet, the protein source or the kaolin. Maltase, the predominant carbohydrase, showed no difference with the altered starch level or the kaolin addition. The decreased activities seen in the groups fed the soyflour and lupin based diets as compared with the group fed the starch based diets must therefore be the result of the protein source. Some factor(s) within these protein sources appears to have an inhibitory effect on the maltase activity. Sucrase showed

significant increases in activity in all reduced starch diets, which in this case must be attributable to the reduction in starch or the kaolin inclusion. If this was a result of the starch reduction it could be said that the abalone are increasing the synthesis of the enzymes to ensure enough carbohydrate nutrient is available for uptake. Following this argument it might be expected that β-galactosidase activity would also increase however this was not the case. With an increase in sucrase activity in the reduced starch groups there was a concurrent decrease in β-galactosidase.

From previous studies (Chapter 3 section 3.5.2 & Chapter 4 section 4.5.4) β-galactosidase was seen to increase as mucus cover increased in the intestine. Here in this study we may be seeing a reduction of mucus caused by the abrasive effect of kaolin, which would allow the enzymes more ready access to the nutrients in the lumen. The reduction in pregelled starch from the levels seen in the soyflour (44%) and lupin (37%) based diets to the level in the same diets with reduced starch (10% in each case) indicate an inhibitory effect on sucrase activity at these higher inclusion levels. In rainbow trout (*Oncorhynchus mykiss*), digestibility studies using pregelled corn starch at different levels, showed a reduction in digestibility and animal growth when pregelled starch was included at 41% in a diet as compared with 11 and 21% (Pfeffer *et al.*, 1995). Other researchers found similar findings with gelatinised wheat starch in trout (Henrichfreise and Pfeffer, 1992) and precooked potato starch in cod (Hemre *et al.*, 1979). It is difficult to distinguish the most likely cause in this case as both the addition of kaolin and the reduction in pre-gelled starch may have an affect on the carbohydrase activity.

The L and the LR group showed a smaller change in activities in all enzymes and may be related to the intestinal morphological changes seen in section 1 where no spherule structures were seen in the previous study (Chapter 4 Plate 4.7a). It was suggested in the previous study (Chapter 4 section 4.5.1.1) that this may locate the main functional activity to section 2 of the intestine and we may be seeing an indication of this by fact that AP and LAP do not change and there is only a small change in comparison to the SR and STR groups for sucrase and β-galactosidase. These enzymes may be at maximal activity for these particular groups.

5.5.2 Reduced Legume Inclusion and Altered Starch:

Alkaline Phosphatase and Proteases.

The addition of soyflour to the diets (13-15%) has most likely caused the increase in alkaline phosphatase activity in the PRI and the BRI groups as the increased activities were similar to that of SR (figure 5.1). LAP activity follows a similar pattern in that PRI and BRI increase

activity and may be attributable to the soyflour, as it has high protein content, or a reduction in the inclusion levels of the legume (field peas 74% to 39% of the diet, faba beans 66%-39%). In rainbow trout fed diets based on peas (*Pisum salivum*) and beans (*Vicia faba*) with differing inclusion levels of 50 and 25% showed that both energy and protein digestibility decreased at the higher inclusion level (Pfeffer *et al.*, 1995). When we consider the S and V groups LAP activity they are both low in comparison to the other groups however in combination in the reduced vetch diet the VRI group LAP activity increases. Arnt *et al.* (1999) found in Coho Salmon (*Oncorhynchus kisutch*) that inclusion levels of 15 to 30% of soyflour resulted in a stepwise reduction in weight gain over a control diet with no soyflour. Protein and energy digestibility efficiencies are related to both the final digestion and uptake of the nutrients. The increased enzyme levels seen in the VRI group in this current study reflect an increased rate of digestion and additional nutrients available for uptake. The result of the above studies are not reflected in abalone in this current study suggesting that the most likely factor influencing the LAP results for PRI and BRI would be the reduced inclusion level of the legume.

Carbohydrases.

The carbohydrase results reflect what was seen with the soyflour and lupin based diets in that maltase had not changed and the increase in sucrase was accompanied by a decrease in β-galactosidase. The difference in the legume source is seen in the maltase results where peas have the higher maltase activity. Both vetch and beans are considered to have more antinutrient factors (ANFs) than does peas (Petterson and MacIntosh, 1994) and it is probable that these effects are seen in the activity levels here. It is possible that even at the reduced inclusion level of beans, ANFs may still be having a major effect on the carbohydrate final digestion and hence the lack of change in enzyme activities seen. The effective increase in amylopectin from pregelled starch addition provides similar levels (27.5 – 33%) in each diet. There is a trend seen with the maltase results when a comparison is made between the P and PRI and the B and BRI groups in that the SA of each legume groups shows no significant difference, however the TA shows a significant decrease in the PRI and BRI groups. This decrease ii likely caused by the increased inclusion of the more digestible pregelled starch resulting in less enzyme protein needed in the tissue to perform final digestion. Effectively there appears to be little inhibition of enzyme function. In the vetch based diets the reduced inclusion levels of starch shows an increase in maltase TA and is possibly the combination of the soyflour and vetch which would increase the complex mix of ANFs in the diet. The

animals are most likely producing more enzyme protein to counter the ANF inhibition of enzyme function.

The effect of reducing the legume source cannot be discounted. The LAP results show no change in TA for the peas and beans groups but a significant increase in SA in the PRI and BRI groups. The reduction in legume and the concurrent SA increase suggests that there is less inhibition of enzyme function from ANF's from the legume source. In comparison the vetch diet groups shows a significant increase in LAP TA in the VRI group and a significant increase in SA however not of the magnitude of the TA results. The inclusion of soyflour has likely increased the ANFS in this diet and the animals are most likely producing more enzyme protein to counter the ANF inhibition of enzyme function as seen with the maltase results.
The results of the other studies mentioned above show considerable improvement in energy digestibility as a result of reducing the inclusion level of the legumes.

5.6 Conclusion:

The inclusion of pregelled starch at levels of 37-43% used in this study reduces carbohydrase activities in the intestine of abalone compared to inclusion levels of 10-25%. The effect of the inert filler kaolin, which in this case was included to reduce the starch level of the diet, may also have had an abrasive effect causing a reduction in the thickness of the mucus layer. This would allow nutrients ready contact with the apical membrane that would increase all activity of the enzymes tested with the exception of β-galactosidase. β-galactosidase decrease in enzyme activity has been shown to reflect the mucus cover (Chapter 4). The effects of inert fillers should be considered in future work. The type of starch also effects carbohydrase activity with higher ratios of amylopectin: amylose levels in diets showing greater activity. The complex mix of native starches of field peas, faba beans and vetch may not be fully digested by abalone. Undigested starch may have ramifications in farming systems by providing substrates for bacterial growth in tanks. The effect of the reducing the legume source in field pea and vetch based diets have shown a concurrent potential decrease in the inhibition of enzyme function. The effects seen on the improvements in carbohydrase activity with dietary starch type would mean that inclusion levels could not be considered wholly responsible for these carbohydrase changes. Faba beans and vetch require more study as to the effects of inherent anti nutrient factors before consideration is made for their use as protein and carbohydrate sources in abalone diets. Peas in abalone diets may be considered although they would need to have another protein source included such that their inclusion level is reduced.

Chapter 6

A Preliminary Study on the Effect of Inert Fillers on the Abalone *Haliotis laevegata* Intestinal Digestive Enzyme Function.

6. A Preliminary Study on the Effect of Inert Fillers on the Abalone *Haliotis laevegata* Intestinal Digestive Enzyme Function.

6.1 Abstract:

The use of naturally occurring clay or other mineral materials as inert fillers in aquaculture diets has shown improvement in growth and can be related to increased digestibility of the feedstuff.

The purpose of this study was to observe if abalone intestine digestive enzyme activity can be stimulated with the inclusion of inert fillers in diets. A diet formulated by the Fisheries, Research and Development Corporation (FRDC) was used as a control. The inert fillers bentonite, diatomaceous earth, sand and kaolin were included at 5% as a partial substitution for semolina in this diet. Kaolin was tested at 5, 10, 15 and 20% inclusion levels.

The analysis showed bentonite and kaolin to increase abalone intestinal enzyme activity. Diatomaceous earth reduced intestinal enzyme activity. Kaolin at 10% inclusion provided the greater overall increase in enzyme activity

Kaolin addition to abalone diets, with a limit of 10%, is recommended as it stimulates enzyme activity. Bentonite would need further testing at increased levels. The use of inert fillers may have additional beneficial effects in farming systems in terms of water purification and waste disposal and deserves further investigation.

6.2 Introduction:

Plant components that are not digested by the endogenous secretions of the gastrointestinal tract are generally referred to as dietary fibre. In aquaculture dietary fibres have been used as non-nutritive fillers or binders in feeds. Their use has met with varied success where incorporation levels above 10% have resulted in growth depression, reduction in feed intake and lowered digestibility of protein and lipid in tilapia (Dioundick and Stom, 1990; Shiau *et al.*, 1988; Storebakken, 1985). However, in other aquatic species, seabass and rainbow trout, high levels of cellulose (15-30%) have not effected growth performance (Bromley and Adkins, 1984; Dias *et al.*, 1998). These contradictory results suggest species differences in terms of digestion and utilization of nutrients thus affecting overall performance.

In animal nutrition, besides fibres, other inert substances such as the silicates bentonite, kaolin, zeolites, diatomides and cement kiln dust have been used as non-nutritive fillers (Edsall and Smith, 1990; Grove *et al.*, 1978; Reinitz, 1984; Rumsey, 1981). Some researchers have found beneficial effects on growth, feed efficiency and nutrient utilization (Mumpton and Fishman, 1977) while others have found negative responses to inert filler use in diets (Sellers *et al.*, 1980; Shurson *et al.*, 1984). In aquaculture the properties of these non-nutritive fillers, such as ion-exchange and absorption, have been exploited to make more efficient use of nitrogen in nutrition to control moisture and ammonia content of faeces, to purify recirculating hatchery waters and to reduce the nitrogen content of hatchery waste water (Mumpton and Fishman, 1977).

The use of sodium bentonite (up to 10%) and zeolites (2.5 and 5%) in tilapia diets has shown improved growth and feed efficiency (Dias *et al.*, 1998) however the use of 5 and 10% zeolites did not effect the growth of Coho salmon (Edsall and Smith, 1990). In rainbow trout Reinitz (1984) found that additions of 5, 10 and 15% sodium bentonite reduced weight gain. The reasons for the improved performance is still not fully understood however the responses have been attributed to a reduced rate of passage of feed through the digestive tract, a buffering action of endogenous particles and nutrients available from the source (Smith, 1980).

In a previous study (Chapter 5 section 5.6) it was difficult to isolate the effects of kaolin on the abalone intestinal digestive enzymes from that of reduced starch levels. There may be benefits to abalone nutrition with the addition of inert fillers if a reduced rate of passage through the intestine is seen. The extra time available for enzymes to complete breakdown of dietary nutrients may improve feed efficiency. Additional benefit may come in waste management in farmed systems by reducing excess dietary ammonia from recirculating water systems and runoff waters. In the present study a commercial style diet was used such that any benefit seen would replicate that which may be seen in farms. The diet used was a formulation from the Fisheries, Research and Development Corporation (FRDC) as it is the basis of most commercial diets in Australia.

There were two objectives to this study;

1. Does the inclusions of inert fillers in diets for abalone stimulate the digestive enzyme activity in the intestine of abalone. The FRDC diet was used as a control. The treatment

diets were this diet with a 5% substitution of the inert fillers, bentonite, sand, diatomaceous earth and kaolin with a subsequent reduction of 5% in semolina. Another diet with a 5% substitution of pregelled waxy maize starch for the semolina in the FRDC diet was formulated as a second control such that comparison could be made to the previous starch experiment.

2. Does increasing levels of inert filler enhance digestive enzyme activity in the intestine of abalone. The above FRDC diet was used as a control with treatment diets using kaolin at 5, 10, 15 and 20% inclusion with equal reductions in semolina.

6.3 Methods:

6.3.1 Animals:

The abalones used in this experiment were from stocks held at South Australian Research and Development Institute (SARDI) West Beach. The abalone stocks held at SARDI were originally obtained from a commercial hatchery. Thirty-six animals were used for each treatment group. Within each treatment group, nine of the abalone were used for histological studies and twenty-seven were used for biochemical analysis.

6.3.2 Diets and Feeding.

The FRDC base diet formulation and inert filler treatments are shown in appendix A Table A5. The alterations for the treatment diets were to reduce the semolina amount by a similar percentage as the inclusion level of inert fillers. Animals were fed to excess daily at 1700hrs for 14 days.

6.3.3 General Methods.

The general methods used in this study are detailed in Chapter 2 (General Materials and Methods) reference numbers are shown.

Aquarium System.	2.1
Abalone Tissue Sampling.	2.2
Apical Membrane Preparation.	2.5
Enzyme Assays.	
Alkaline Phosphatase	2.6.1
Maltase, sucrase and β-galactosidase	2.6.2
Leucine Amino-peptidase	2.6.3
Succinate dehydrogenase	2.7.1
Acid Phosphatase	2.7.2

Sodium/potassium ATPase	2.8
Protein.	2.9

6.3.4 Chemicals.

Glucose determination used a Boehringer-Mannheim test kit (Chromogen - ABTS) purchased from Adelab Adelaide, all other chemicals were the best available analytical grade and purchased from Sigma (Sydney Australia).

6.3.5 Statistical Analysis

The data are expressed as means ± SE. Significant differences between means were calculated with one-way analysis of variance on Genstat version 5 ($P<0.05$).

For simplicity in referencing, abalone fed a diet with an addition of a particular inert filler; the abalone intestine will be referred to after the inert filler and its inclusion level. For example bentonite (B5), the intestine of abalone fed the FRDC diet with inclusion of 5% bentonite. For abalone fed diets with varying inclusion levels of kaolin, the abalone intestine will be referred to after the inclusion level i.e. the intestine of abalone the FRDC diet with a 10% kaolin inclusion (K10).

Filler	Diet
FRDC	FRDC
Starch	ST5
Bentonite	B5
Sand	S5
Diatomaceous Earth	DE5
Kaolin 5, 10,15, 20%	K5, K10, K15, K20

6.4 Results:

Apical Membrane Isolation

The apical membrane isolation procedure had been successful and would be suitable for studying the effects of dietary components on enzymes in this study. Protein recovery for the apical membrane fraction was between 7.1 and 11.5% of the protein present in the original homogenate.

6.4.1 Inert Fillers 5% Inclusion:

Alkaline Phosphatase.

The inclusion of sand, bentonite and kaolin in comparison to the FRDC base diet significantly reduced the intestinal alkaline phosphatase activity (9-37%) in abalone fed diets with these inert fillers included. The ST5 and DE5 groups show no significant difference with the exception of the total activity of the ST5 group, which is reduced (8%) (Figure 6.1).

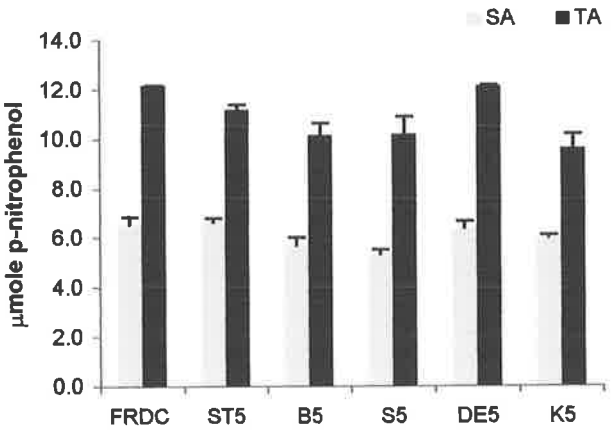


Figure 6.1: Effect of Inert Fillers on Intestinal Alkaline Phosphatase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevegata.*

Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity (SA) is expressed as µmole p-nitrophenol/mg protein/hr, total activity (TA) is expressed as µmole p-nitrophenol/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. In comparison to the FRDC base diet for SA treatments B5, S5 and K5 were significantly different at $P<0.05$. In comparison to the FRDC base diet for TA, treatments ST5, B5, S5 and K5 were significantly different at $P<0.05$. FRDC base diet (FRDC), following diets FRDC with 5% inclusion of the inert filler or starch, (ST5) starch, (B5) bentonite, (S5) sand, (DE5) diatomaceous earth, (K5) kaolin.

Carbohydrases.

Relative ranking of carbohydrase activity in the intestine showed maltase>sucrase>β-galactosidase (Figure 6.2). Maltase activity is not changed with inert filler inclusion except for the specific activity of the DE5 group (reduced 13%) and the total activity of the K5 group (reduced 16%). Sucrase activities significantly increase with the B5 and S5 groups. The sucrase specific activity of the K5 group increases (29%) as does the total activity of the DE5 group (19%). β-galactosidase activity significantly reduces in the B5, S5 and DE5 groups. The specific activity of the ST5 group is significantly reduced (14%) as is the total activity of the K5 group (19%).

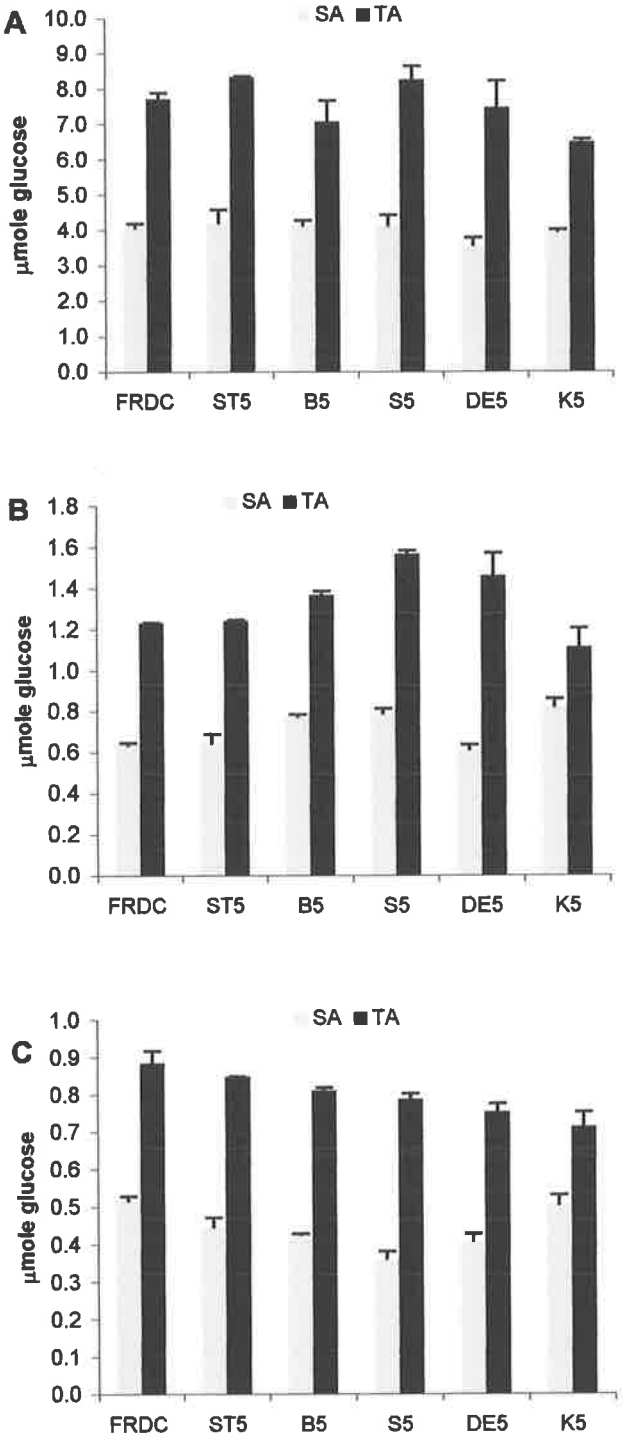


Figure 6.2: Effect of Inert Fillers on Intestinal Carbohydrases Maltase (A), Sucrase (B) and β-galactosidase (C) Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole glucose/mg protein/hr; total activity (TA) is expressed as µmole glucose/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. In comparison to the FRDC base diet for maltase SA DE5 and for TA K5 were significantly different at $P<0.05$. In comparison to the FRDC base diet for sucrase SA B5, S5 and K5 and for TA S5 and DE5 were significantly different at $P<0.05$. In comparison to the FRDC base diet for β-galactosidase K5 and for TA ST5 were not significantly different at $P<0.05$. FRDC base diet (FRDC), following diets FRDC with 5% inclusion of the inert filler or starch, (ST5) starch, (B5) bentonite, (S5) sand, (DE5) diatomaceous earth, (K5) kaolin.

Protease.

The leucine amino-peptidase (LAP) specific activity of is significantly reduced in the ST5, S5 and DE5 groups (12, 4, and 19% respectively) while the B5 and K5 groups show significant increases (6 and 7% respectively) (Figure 6.3). In addition the total activity is significantly reduced in the K5 group (15%) and significantly increased in the DE5 group (13%).

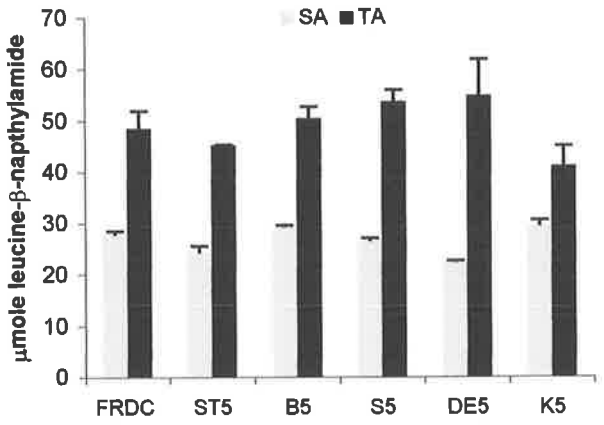


Figure 6.3: Effect of Inert Fillers on Leucine Amino-Peptidase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole leucine-β-napthylamide/mg protein/hr, total activity (TA) is expressed as µmole leucine-β-napthylamide /gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. In comparison to the FRDC base diet for SA all treatments and for TA DE5 and K5 were significantly different at $P<0.05$.. FRDC base diet (FRDC), following diets FRDC with 5% inclusion of the inert filler or starch, (ST5) starch, (B5) bentonite, (S5) sand, (DE5) diatomaceous earth, (K5) kaolin.

6.4.2 Increasing Levels of the Inert Filler Kaolin:

Alkaline Phosphatase.

At all kaolin inclusion levels there is a significant reduction in alkaline phosphatase activity, K10, K15 and K20 showing the greater change (Figure 6.4).

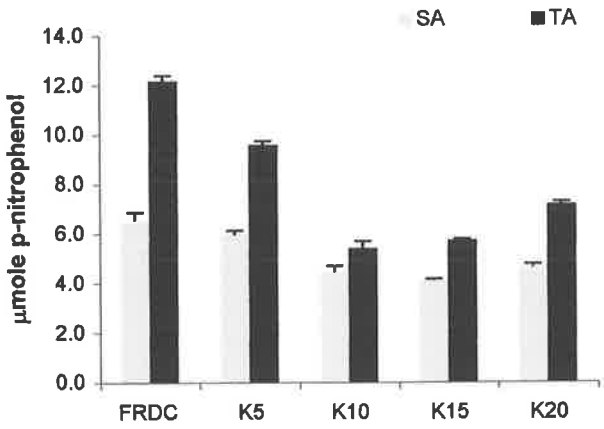


Figure 6.4: Effect of Increasing Levels of the Inert Filler Kaolin on Intestinal Alkaline Phosphatase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevegata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity (SA) is expressed as µmole p-nitrophenol/mg protein/hr, total activity (TA) is expressed as µmole p-nitrophenol/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. In comparison to the FRDC base diet for SA and for TA all treatments were significantly different at $P<0.05$. FRDC base diet (FRDC), K5, K10, K15 and K20 denotes the FRDC base diet with 5, 10, 15 and 20% inclusion of kaolin respectively.

Carbohydrases.

The K10 group maltase specific activity is significantly greater than the FRDC control while the K15 group specific activity is significantly reduced (11%) (Figure 6.5). The total activity reduces stepwise with increasing kaolin levels K5-K15 groups and at 20% inclusion of kaolin the K20 group increases but not to the same level as the control. Sucrase activity in the abalone intestine shows a stepwise increase in specific activity with increased kaolin levels. The total activity is reduced in the K10 group (23%) and increased in the K20 group (43%). β-galactosidase activity is significantly reduced in the K10 and K15 groups with the specific activity of the K20 group increasing significantly (16%).

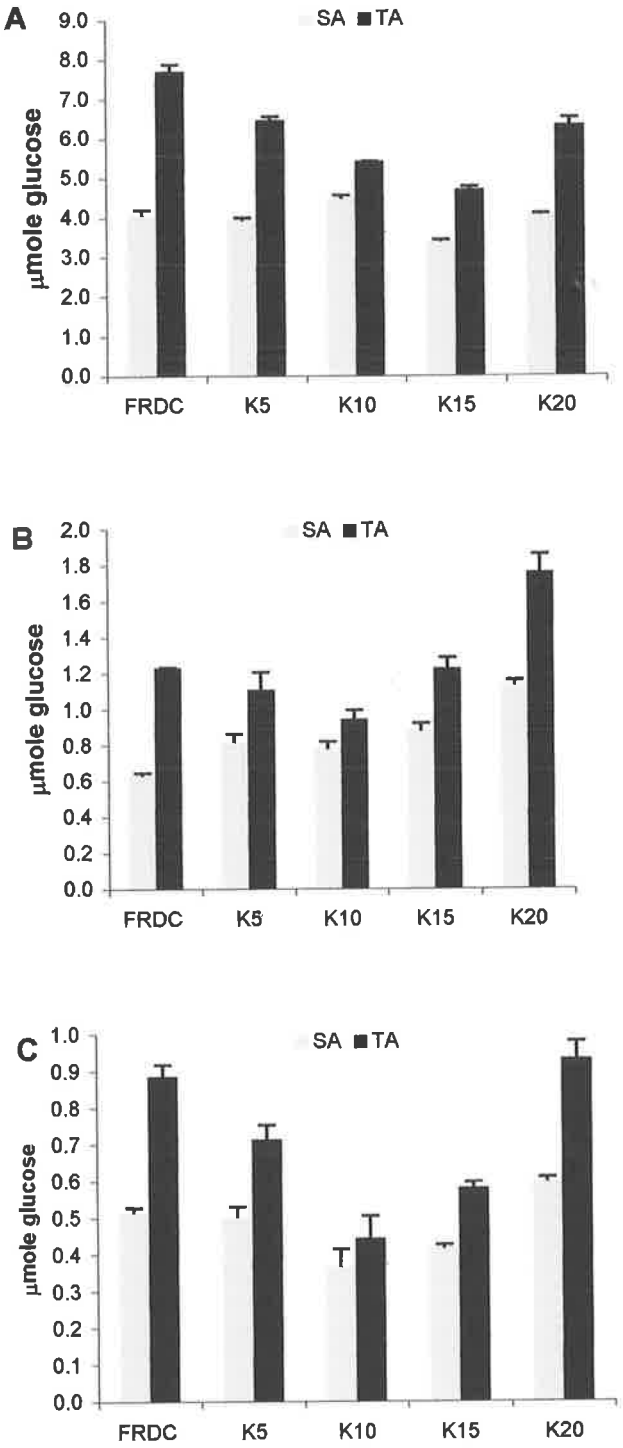


Figure 6.5: Effect of Increasing Levels of the Inert Filler Kaolin on Intestinal Carbohydrases Maltase (A), Sucrase (B) and β-galactosidase (C) Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole glucose/mg protein/hr; total activity (TA) is expressed as µmole glucose/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. In comparison to the FRDC base diet for maltase SA K5 and K20 and for TA K20 were not significantly different at $P<0.05$, for sucrase SA all treatments, and for TA K10 and K20 were significantly different at $P<0.05$. In comparison to the FRDC base diet for β-galactosidase K5 and for TA K20 were not significantly different at $P<0.05$. FRDC base diet (FRDC), K5, K10, K15 and K20 denotes the FRDC base diet with 5, 10, 15 and 20% inclusion of kaolin respectively.

Protease.

The K5 and K20 groups show significant increases in leucine amino-peptidase specific activity (figure 6.6). The leucine amino-peptidase total activity is significantly reduced in the K5, K10 and K15 groups.

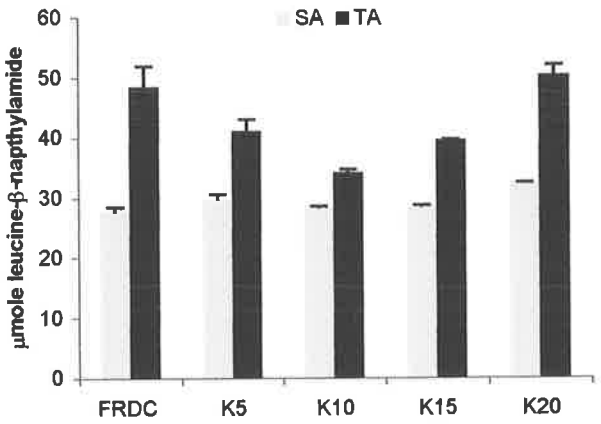


Figure 6.6: Effect of Increasing Levels of the Inert Filler Kaolin on Leucine Amino-Peptidase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole leucine-β-napthylamide/mg protein/hr, total activity (TA) is expressed as µmole leucine-β-napthylamide /gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. In comparison to the FRDC base diet for SA K5and K20 and for TA K5, K10 and K15 were significantly different at $P<0.05$. FRDC base diet (FRDC), K5, K10, K15 and K20 denotes the FRDC base diet with 5, 10, 15 and 20% inclusion of kaolin respectively.

6.5 Discussion:

6.5.1 Inert Filler 5% Inclusion.

The inclusion of inert fillers at a 5% level has led to differences in digestive enzyme activity in abalone intestine. Abalone fed diets with inclusions of bentonite and kaolin have shown an overall increase in intestinal enzyme activity while abalone fed the diet with diatomaceous earth show a general decrease in intestinal activity.

The use of diatomaceous earth reduced both protein and carbohydrase activity and appears to have little value as an inclusion to stimulate intestinal enzyme activity, in fact it has increased the TA for all enzymes tested except maltase indicating an inhibitory effect on these enzymes and an increased production of enzyme protein. It is most likely that greater undigested feed would be excreted causing increased nitrogen or ammonia in recirculating water systems and in effluent water (Edsall and Smith, 1990; Mumpton and Fishman, 1977). Ammonia levels,

lower dissolved oxygen and pH have all been shown to reduce growth and viability of abalone *Haliotis laevegata* (Harris *et al.*, 1996; Harris *et al.*, 1998; Harris *et al.*, 1999).

The inclusion of pregelled starch at this 5% level has had no beneficial effect on the abalone intestinal enzyme activity as overall there is no difference in activity to the control FRDC group. Leucine amino-peptidase activity (LAP) is reduced and it would be doubtful that this would be because of a reduction in protein. Semolina does have a 12% protein component (Vanderpeer, 2001) and would be reduced with the pure starch included however the protein of this diet would not become limiting with this minor change (0.6%). High inclusion levels of gelatinised starch have also been shown to reduce digestibility of protein and energy and effect growth in rainbow trout (Henrichfreise and Pfeffer, 1992; Pfeffer *et al.*, 1995) and cod (Hemre *et al.*, 1979). At this inclusion level of pregelled starch we would not see this effect as the carbohydrases show little change.

The beach sand particles used in the sand diet were more granulated than the other inert fillers used as processing has turned these fillers into a powder. The effect of the sand may be seen in the reduced β-galactosidase activity. β-galactosidase has been related to the amount of mucins in the intestine in a previous study (Chapter 4 section 4.5.4), indicating that the more abrasive effect of the sand, in comparison to the other inert fillers, may be removing a greater portion of the mucus layer. Sand inclusion may also be slowing the transit time of feed in the gastrointestinal tract due to the physio-chemical properties of ion binding and water holding capacity (Dias *et al.*, 1998). With these properties, and in combination with the reduced mucus layer, we see increases in sucrase activity and a much-decreased alkaline phosphatase (AP) activity. In a previous study (Chapter 4 section 4.5.2.2), inclusion of a bacterial phytase enzyme reduced AP enzyme and was likely due to the release of bound phosphate in phytic acid. In this study there may be a similar effect however here it would be due to the abrasive effect of the sand mechanically breaking down the feedstuff, mainly in the crop, rather than an enzyme action.

Bentonite and kaolin, both show a significant stimulation of most intestinal digestive enzymes when included in diets for abalone. The major difference between the two is the increase in TA in B5 in comparison to K5 suggesting greater enzyme protein production. The stimulation of the specific activity in the K5 group for all enzymes tested and the reduced total activity suggests that there is a greater and more 'effective' final digestion of feedstuffs, as there is little inhibition of enzyme function and no need for synthesis of additional enzyme protein to

counter any losses. When we relate this to a farmed situation less undigested feed would be in the tank systems therefore the wastes are less likely to cause health or water contamination problems.

6.5.2 Increasing Levels of the Inert Filler Kaolin:

There are several possibilities for the effects seen in this section of the study with kaolin inclusion at 10% and greater. These are an abrasive effect; slower transit time of nutrients through the gut and less antinutrient factors in the diet.

An increased abrasive effect, similar to that seen in the S5 group discussed above, may be mechanically breaking down the feedstuff with the increased levels of kaolin introduced into the diets and would be in combination with enzymes. It is likely that this abrasive effect is releasing bound phosphorous from feed components and hence the reduction in alkaline phosphatase. The reduced alkaline phosphatase SA and TA suggests that there is not as much requirement for this enzyme to provide this role. Another possible indicator of the abrasive effect is the β-galactosidase activity, which shows greater decreases with the K10 and K15 groups, and may be related to less mucus in the intestine. There is, however, a significant increase in β-galactosidase activity with the K20 group, which may indicate that the abrasive effect is too severe and the abalone is secreting more mucins to protect the intestinal lining.

The physio-chemical properties of kaolin such as the ion binding and water holding capacity would be increased and effectively slow the transit time of nutrients in the intestine (Dias *et al.*, 1998; Storebakken, 1985; Wood, 1974). These properties have a strong influence on solubility, gelling and viscosity of food during its passage through the gastrointestinal tract (Storebakken and Austreng, 1987). The slower passage would allow enzymes to interact with their substrates for a greater period of time leading to a more complete breakdown of feed and hence more effective enzyme activity. The general decrease in TA for all enzymes in the K5, K10 and K15 groups suggests less enzyme protein production to achieve breakdown of feedstuffs.

Semolina is known to cause problems in poultry due to soluble non starch polysaccharides such as β-glucans and arabinoxylans which elicit negative effects through increasing viscosity (Annison, 1993; Choct *et al.*, 1996; Choct and Annison, 1992; Dusel *et al.*, 1997). High gut viscosity decreases the rate of diffusion of substrates and hinders their effective interaction

with digestive enzymes at the mucosal surface (Choct, 1997). A reduction in activity of digestive enzymes through direct complexing of viscous polysaccharides has also been suggested (Ikeda and Kusano, 1983). With the greater reduction in semolina in these diets we may be seeing less inhibitory effect of the antinutrient factors known in semolina on the digestive enzymes. This is reflected in the general reduction in TA results of abalone fed these diets with increased kaolin, in comparison to the FRDC control group, which indicates less enzyme protein is needed to achieve digestion. The reduced AP activity in the K10 to K20 groups may be due to less stressed animals as a result of the decreased semolina. Stress is known to increase AP activity (Mizuno *et al.*, 1992). The general decrease in TA of enzyme activity is greatest with the K10 group suggesting this group use less enzyme protein to achieve a similar enzyme SA . We can make the same conclusion as above in that with the addition of kaolin there is a greater final digestion of feedstuffs however at inclusion levels above 10% it appears that no substantial gain is made.

6.6 Conclusion:

This study is a preliminary approach to the use of naturally occurring inert fillers in the diets of abalone. The addition of kaolin and bentonite as inert fillers show decreased TA of intestinal enzymes suggesting that this group had a more efficient enzyme capacity in that less enzyme protein was needed for digestion. The inclusion of kaolin at 10% showed the greater effect and levels above this are not recommended. Bentonite should be tested at increased inclusion levels to see if further benefit can be made at higher levels as it did improve enzyme activity at the 5% inclusion level. A major benefit to farms for the use of inert fillers in diets may be in the water quality of tanks and effluent systems. Kaolin or bentonite would pass through the digestive tract and be excreted in the faeces where it would become available for ammonia absorption in the water. Any uneaten feed at the bottom of the tank would also contain these fillers and any ion exchange with ammonia would take place there. To fully evaluate the use of dietary fillers, aspects concerning the removal of wastes in farm recirculating water systems and effluent waters deserve further investigation.

Chapter 7

Carbohydrate Binding and the Agglutinating Activity of Antinutrient Factors of Various Legumes Sources on the Intestine of the Abalone *Haliotis laevegata*.

7. Carbohydrate Binding and the Agglutinating Activity of Antinutrient Factors of Various Legumes Sources on the Intestine of the Abalone *Haliotis laevegata.*

7.1 Abstract:

Some antinutritional components of legumes are known to readily bind to one or more carbohydrate moieties leading to cell agglutination. This binding can cause toxic effects in animals such as impairing intestinal apical membrane enzyme activity and nutrient uptake.

The carbohydrate-binding and cell agglutination properties of four legumes, the field pea (*Pisum sativum* cv. Alma), yellow lupin (*Lupinus luteus*), faba bean (*Vicia faba* cv. Flord) and vetch (*Vicia sativa* cv. Blanchefleur) were tested on isolated intestinal apical membranes from wild (WRA) and commercially reared (CRA) abalone *Haliotis laevegata.* Treatments were raw and dry (oven) and moist (autoclaved) heat treatments at different cooking times. A feed trial was conducted with diets based on dry heat treated peas (100°C 10 minutes) and autoclaved lupins (120°C 5 minutes). A biochemical analysis of intestinal enzyme function was studied.

The heat treatment of beans and vetch show little differences in cell agglutination activity from isolated intestine of WRA. Peas show less agglutination activity with dry heat treatments while lupins show less agglutination activity with autoclaved treatments in WRA. CRA show little change in agglutination activity in peas with dry heat at 100°C for 10 minutes duration, all other time/temperature regimes increased cell agglutination. A reduction in cell agglutination was seen in all heat treatments of lupins using CRA intestine. A field trial using commercially reared abalone shows minor positive effects on intestinal enzyme function with dry heat-treated peas in diets and a reduced effects with moist heat-treated lupins in diets.

The agglutination assay shows promise in predicting the influence of components of legumes on intestinal enzyme function in their raw and treated states. Further work is required at different time/temperature regimes, which may lead to improvement in diet quality and cost savings in the manufacture of diets.

7.2 Introduction:

Isolated intestinal apical membranes have been used over the past two decades to study both structure function relationships, transport and chemiosmotic properties of cell membranes

(Christensen, 1989; Kilberg *et al.*, 1993; McGiven and Pastor-Anglada, 1994; Sachs *et al.*, 1980; Stevens *et al.*, 1984). They have been used as a tool in nutritional studies where inferences about the effects of dietary components have been made by testing enzyme activity in these membranes and noting changes that occur (Bamba *et al.*, 1990; Gawlicka *et al.*, 1996).

More recently they have been used to determine the carbohydrate-binding and agglutinating effect of lectins on the intestinal apical membrane (Grant and van Driessche, 1993; Irish *et al.*, 1997; Irish *et al.*, 1999; Maenz *et al.*, 1999). Plant lectins are dimers or tetramers with multiple carbohydrate binding sites that have a potential to agglutinate components of cell membranes. Depending on the match between the carbohydrate specificity of the lectin and the glycosylation pattern of the cell membrane, lectins can associate with two or more carbohydrate moieties, leading to a cell agglutination (Irish *et al.*, 1999). The binding and agglutinating properties of plant lectins can lead to toxic effects in animals such as shortening of the villus structure, increased cell turnover and impaired apical membrane enzyme and uptake function (Grant and van Driessche, 1993), however the antinutritional effects of dietary lectins require an initial binding to carbohydrates on the apical membrane of the intestinal cells (Pusztai *et al.*, 1990). In rat small intestine, there is considerable *N*-actylgalactosamine and *N*-acetylglucosamine sites on the glycoproteins in the apical membrane (Irish *et al.*, 1999). Lectins from soybean, kidney bean and wheat germ bind readily to these carbohydrates and are toxic when consumed in their native state. In contrast, faba bean, lentil and pea lectins have a poor affinity to rat intestine and are non-toxic when fed to rats (Grant and van Driessche, 1993). Denaturation of the lectins by heat treatment may disrupt the quaternary and tertiary structures and reduce the functional capacity to agglutinate cell membranes (Liener, 1994). There are some plant lectins however that are resistant to inactivation by dry heat, soybean being one (De Muelenaere, 1964). The degree and duration of heat exposure and moisture levels in the processing of soyflour has shown that at shorter duration and severity of heat treatment more significant levels of lectins are found with antinutritional properties (Maenz *et al.*, 1999).

In a previous study (Chapter 4 section 4.5.3.2), which looked at heat treatment of legumes, the abalone intestinal carbohydrases showed an increase in abalone fed diets of peas and vetch, which had been autoclaved prior to inclusion in the diet. It was suggested that due to the high levels of starch in the legumes the starch had been gelatinised by autoclaving, hence making it more digestible, or that antinutritional factors (ANFs) had been denatured lessening or

removing their effect. It may be possible that lectins or some other components of the legumes were having a carbohydrate binding or agglutination effect, which may have been reduced by heat treatment. Given the result there may be value in testing a range of dry and autoclaved heat treatments on the legumes to see if potential binding and agglutination effects can be reduced. The principle behind the agglutination assays of Irish *et al.* (1999) is that lectin protein is added to isolated apical membranes and incubated for a period of time to allow binding and agglutination to occur. The membranes are then isolated from this medium by centrifugation and assayed for alkaline phosphatase activity. Reduced activity of alkaline phosphatase indicates some degree of carbohydrate binding or agglutination from lectins. In this study, rather than look at specific components, it was decided to use the whole legume seed such that other components in addition to lectins that may be having an antinutritional effect can be assessed. A considerable time saving applied, as the individual antinutrient factors, such as lectins, did not have to be isolated. The legumes lupins, filed peas, faba beans and vetch were used.

There were two objectives to the study;

1. To refine an agglutination assay to measure the capacity of components of legumes to agglutinate isolated intestinal apical membranes (section 1, section 2, total) from wild reared (WRA) and commercially reared (CRA) abalone *Haliotis laevegata*. The results of this experiment were used to select suitable legume/s and heat treatment/s for inclusion in the feed trial on abalone.
2. To feed abalone diets of raw and heat-treated legume/s and test isolated intestinal apical membrane to observe if differences can be found in enzyme activity that may reflect carbohydrate or agglutination of the intestinal apical membrane.

7.3 Methods:

7.3.1 Agglutination Experiment.

7.3.1.1 Heat Treatment of Legumes.

The field pea (*Pisum sativum* cv. Alma), yellow lupin (*Lupinus luteus*), faba bean (*Vicia faba* cv. Flord) and vetch (*Vicia sativa* cv. Blanchefleur) were used in these experiments.

All legumes (whole seed) were ground in a hammer mill and then in a centrifugal lab mill with a 2mm sieve. Samples of 300g of each legume were subjected to the following heat treatments: dry heat for 10 and 30 minutes at 100°C and autoclaving at 120°C, 120-130kPa for

5 and 20 minutes. Additional treatments for peas were dry heat for 10 minutes at 50, 150, 200°C and lupins autoclaved at 120°C, 130kPa for 5 and 12 minutes.

7.3.1.2 Animals

The commercially reared abalone (CRA) abalones used in this experiment were from stocks held at South Australian Research and Development Institute (SARDI) West Beach. The abalone stocks held at SARDI were originally obtained from a commercial hatchery. Wild reared abalones (WRA) were collected from near Port Lincoln South Australia.

7.3.1.3 Diets and Feeding.

Due to the difficulty in getting wild abalone to accept manufactured diets it was necessary to look at possible differences in carbohydrate binding and agglutination activity of legumes on the isolated intestinal membrane of CRA. The CRA abalones were reared on the CRA1 diet as used in Chapter 3 prior to being fed the experimentation diets. Diet formulations are shown in appendix A Table A1 and Table A2. Animals were fed to excess daily at 1700hrs for 14 days.

7.3.1.4 Agglutination Assay.

Legume sample supernatants were placed in a buffer containing mannitol 300mM, tris(hydroxymethyl)aminomethane (Tris) 2mM, $MgSO_4$ 0.1mM and 0.02% NaN_3. Agglutination was initiated by adding 100µg of isolated apical membrane to 400µl of supernatant buffer and incubated for 12 hours at 4°C. Two blanks, one containing 100µg apical membrane the other 100µl of legume supernatant were also incubated for 12 hours at 4°C. The apical membranes were pelleted by low speed centrifugation at 1100rpm for 10 minutes. The pellets were resuspended in a buffer containing mannitol 300mM, Tris 2mM, $MgSO_4$ 0.1mM and 0.02% NaN_3. Alkaline phosphatase (refer chapter 2.6.1) was then determined using agglutinated apical membrane protein levels of 25µg.

7.3.2 General Methods.

The general methods used in this study are detailed in Chapter 2 (General Materials and Methods), reference numbers are shown.

Aquarium System.	2.1
Abalone Tissue Sampling.	2.2
Apical Membrane Preparation.	2.5
Enzyme Assays.	
Alkaline Phosphatase	2.6.1
Maltase, sucrase and β-galactosidase	2.6.2

Leucine Amino-peptidase	2.6.3
Succinate dehydrogenase	2.7.1
Acid Phosphatase	2.7.2
Sodium/potassium ATPase	2.8
Protein.	2.9

7.3.4 Chemicals.

Glucose determination used a Boehringer-Mannheim test kit (Chromogen - ABTS) purchased from Adelab Adelaide, all other chemicals were the best available analytical grade and purchased from Sigma (Sydney Australia).

7.3.5 Statistical Analysis

The data are expressed as means ± SE. Significant differences between means were calculated with one-way analysis of variance on Genstat version 5 ($P<0.05$).

Referencing

For simplicity in referencing abalone fed a diet with a particular treatment, the abalone intestine will be referred to after the source and/or its treatment. For example heat treated peas (PH) the intestine of abalone fed a diet with the dry heat treatment of peas, (LH) the intestine of abalone fed a diet with the autoclaved heat treatment of lupins and (P) or (L) the intestine of abalone fed a diet of peas or lupins respectively with no treatment (raw).

7.4 Results:

7.4.1 Agglutination Experiment

With the exception of vetch there is no significant difference in agglutination activity between section 1 and section 2 of the intestine within each treatment group. In all cases there is a reduction of alkaline phosphatase (AP) in relation to the control with the agglutination activity for lupins>peas> vetch>beans (Figure 7.1).

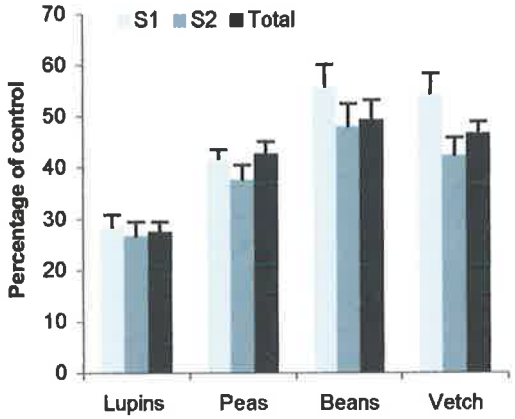


Figure 7.1: Agglutination Activity of Components of Raw Legumes on Isolated Intestinal Apical Membrane of Wild Reared Abalone *Haliotis laevegata*.

Data are the mean ± SE for n=9 with 9 determinations from each pooled animal group for each treatment. Activity is expressed as the percentage of Alkaline Phosphatase specific activity (μmole p-nitrophenol/mg protein/hr) seen in apical membrane with no treatment. Single factor ANOVA was used to assess significant differences for treatment. Within Legume vetch S1 was significantly different to S2 and Total at $P<0.05$. (S1) and (S2) denotes section 1 and section 2 of the intestine, (Total) denotes whole intestine (section 1 and section 2).

The effect of dry and autoclaved heat treatments on agglutination activity of isolated intestine from WRA shows in beans and vetch (Figure 7.2) there is no significant difference in the percentage of the control of AP (%CAP) activity except for dry heat treatment of 100°C for 30 minutes, which is significantly reduced (7% beans 5% vetch). Dry heat treatment of peas shows significant increases in the %CAP activity (14% 10100 and 20% 30100) whereas autoclaving causes a further decrease in %CAP activity (3% A5120 and 7% A20120). Dry heat treatment of lupins has no effect on the %CAP activity however autoclaved treatment of 120°C for 5 minutes causes a significant increase in the activity (22%).

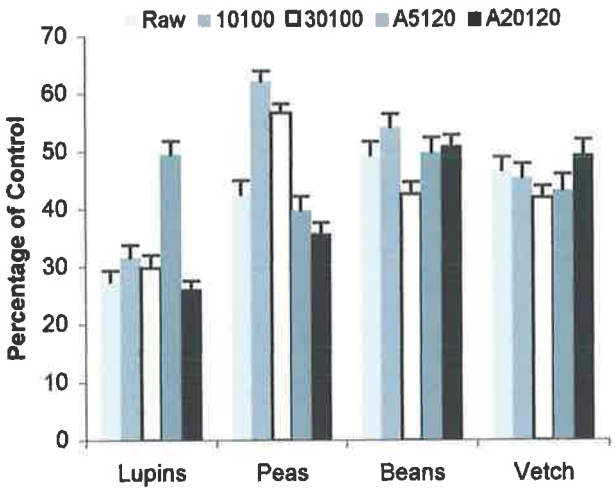


Figure 7.2: Agglutination Activity of Components of Raw and Heat Treated Legumes on Isolated Intestinal Apical Membrane of Wild Reared Abalone *Haliotis laevegata*.

Data are the mean ± SE for n=9 with 9 determinations from each pooled animal group for each treatment. Activity is expressed as the percentage of Alkaline Phosphatase specific activity (µmole p-nitrophenol/mg protein/hr) seen in apical membrane with no treatment. Single factor ANOVA was used to assess significant differences for treatment. Within legume and in comparison to the Raw treatment for lupins A5120, for peas 10111, 30100 and A20120, for beans 310100 were significantly different at $P<0.05$. (Raw) denotes untreated legume, (10100) legume dry heat treated at 100°C for 10 minutes, (30100) legume dry heat treated at 100°C for 30 minutes, (A5120) legume autoclaved at 120°C for 5 minutes and (A20120) legume autoclaved at 120°C for 20 minutes.

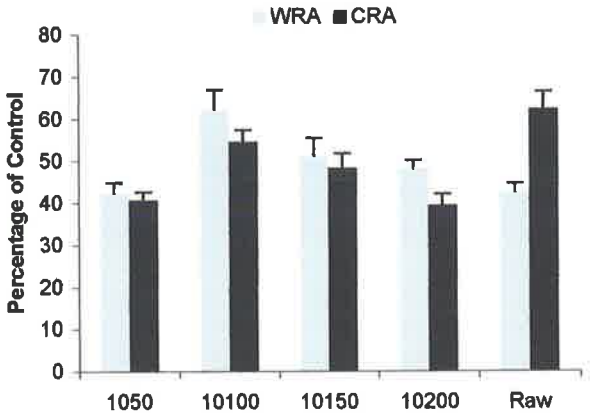


Figure 7.3: Agglutination Activity of Components of Raw and Dry Heat Treated Field Peas *Pisum sativum* on the Intestinal Apical Membrane of Wild Reared and Commercially Reared Abalone *Haliotis laevegata*.

Data are the mean ± SE for n=9 with 9 determinations from each pooled animal group for each treatment. Activity is expressed as the percentage of Alkaline Phosphatase specific activity (µmole p-nitrophenol/mg protein/hr) seen in apical membrane with no treatment. Single factor ANOVA was used to assess significant differences for treatment. Within rearing regimes and in comparison to the Raw treatment for WRA 10100, 10150 and 10200 and for CRA all treatments were significantly different at $P<0.05$. (WRA) denotes wild reared abalone, (CRA) denotes commercially reared abalone. (Raw) denotes untreated peas, (1050) peas dry heat treated at 50°C for 10 minutes, (10100) peas dry heat treated at 100°C for 10 minutes, (10150) peas dry heat treated at 150°C for 10 minutes and (10200) peas dry heat treated at 200°C for 10 minutes.

Comparing the effect of various heat treatments of peas on the agglutination of isolated apical membrane from WRA and CRA (Figure 7.3) shows that all heat treatments follow a similar

pattern with the highest %CAP with peas treated at 100°C for 10 minutes. There is a significant difference with the raw treatment with CRA membranes showing a much greater %CAP (20%) than WRA membranes.

Autoclaving lupins at all times tested shows a decrease in %CAP for CRA (4-8%)(Figure 7.4) whereas WRA at 5 and 12 minutes treatments show a greater increase (20 and 12% respectively) in activity over the raw lupin treatment and a stepwise reduction with increasing time.

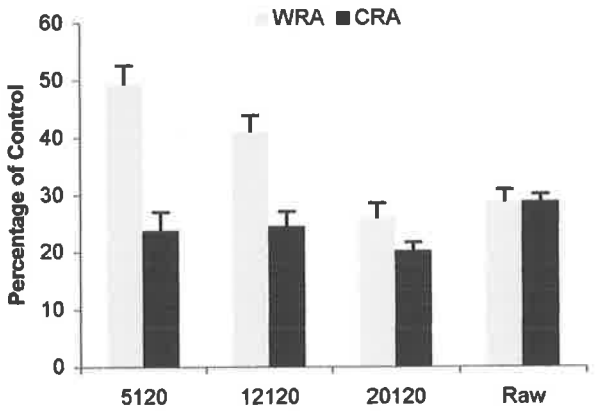


Figure 7.4: Agglutination Activity of Components of Raw and Autoclaved Heat Treated Lupins *Lupinus luteus* on Isolated Intestinal Apical Membrane of Wild Reared and Commercially Reared Abalone *Haliotis laevegata*.

Data are the mean ± SE for n=9 with 9 determinations from each pooled animal group for each treatment. Activity is expressed as the percentage of Alkaline Phosphatase specific activity (μmole p-nitrophenol/mg protein/hr) seen in apical membrane with no treatment. Single factor ANOVA was used to assess significant differences for treatment. Within rearing regimes and in comparison to the Raw treatment for WRA 5120 and 12120 and for CRA all treatments were significantly different at $P<0.05$. (WRA) denotes wild reared abalone, (CRA) denotes commercially reared abalone. (Raw) denotes untreated peas, (5120) lupins autoclaved at 120°C for 5 minutes, (12120) lupins autoclaved at 120°C for 12 minutes and (20120) lupins autoclaved at 120°C for 20 minutes.

7.4.2 Feeding Trial:

Apical Membrane Isolation

The apical membrane isolation procedure had been successful and would be suitable for studying the effects of dietary components on enzymes in this study. Protein recovery for the apical membrane fraction was between 7.2 and 9.4% of the protein present in the initial homogenate.

Alkaline Phosphatase.

Alkaline phosphatase activity (Figure 7.5) significantly decreases with the PH group whereas the LH group shows a decrease in total activity only.

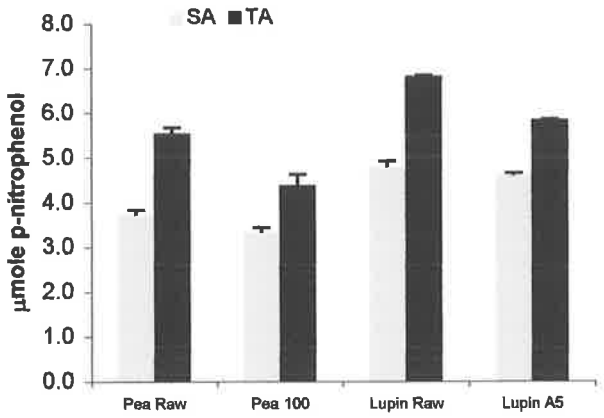


Figure 7.5: Effect of Diets Formulated with Heat Treatments of Peas and Lupins on Intestinal Alkaline Phosphatase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Alkaline Phosphatase specific activity (SA) is expressed as µmole p-nitrophenol/mg protein/hr; total activity (TA) is expressed as µmole p-nitrophenol/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume for the pea treatments SA and TA and for the lupin treatment TA were significantly different at $P<0.05$. (Pea 100) denotes peas dry heat treated at 100°C for 10 minutes, (raw) denotes inclusion of legume with no treatment, (Lupin A5) denotes autoclaved heat treatment of lupins at 120°C for 5 minutes.

Carbohydrases.

Intestinal maltase activity (figure 7.6) shows a significant reduction in total activity in both the PH and LH groups, the specific activity had no change. The PH group shows no change in sucrase activity and a significant decrease in β-galactosidase activity. Sucrase and β-galactosidase activity significantly decreases in the LH group.

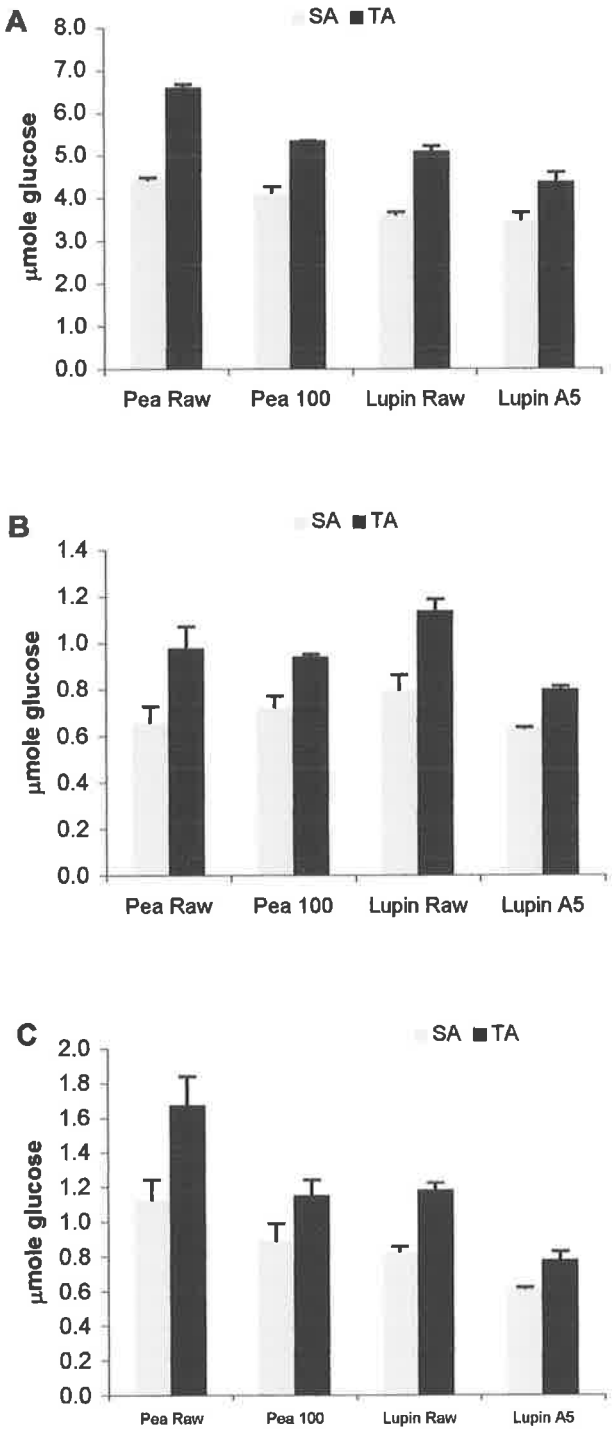


Figure 7.6: Effect of Diets Formulated with Heat Treatments of Peas and Lupins on Intestinal Carbohydrases Maltase (A), Sucrase (B) and β-galactosidase (C) Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole glucose/mg protein/hr; total activity (TA) is expressed as µmole glucose/gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume for both pea and lupin treatments for maltase the TA, for sucrase lupin treatments for SA and TA and for β-galactosidase pea and lupin treatments were significantly different at $P<0.05$. (Pea 100) denotes peas dry heat treated at 100°C for 10 minutes, (raw) denotes inclusion of legume with no treatment, (Lupin A5) and denotes autoclaved heat treatment of lupins at 120°C for 5 minutes.

Protease

Total activity of leucine amino-peptidase (LAP) is not altered in the PH group while the specific activity increases (Figure 7.7). Lap activity is significantly decreased in the LH group in comparison to the L group.

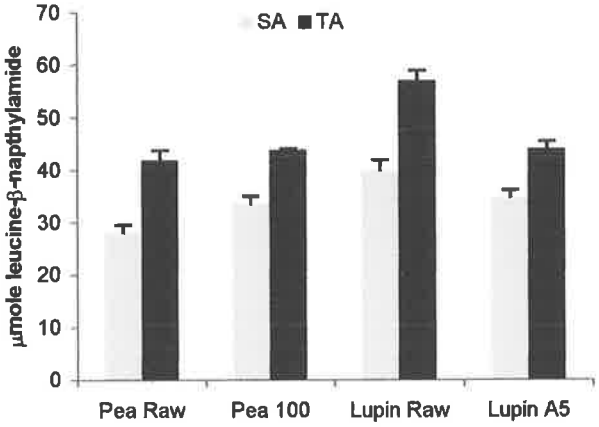


Figure 7.7: Effect of Diets Formulated with Heat Treatments of Peas and Lupins on the Protease Leucine Amino-peptidase Specific Activity and Total Activity in Isolated Intestinal Apical Membrane from the Abalone *Haliotis laevigata*.

Data are the mean ± SE for n=10 with 9 determinations from each animal. Specific activity (SA) is expressed as µmole leucine-β-napthylamide/mg protein/hr, total activity (TA) is expressed as µmole leucine-β-napthylamide /gm tissue. Single factor ANOVA was used to assess significant differences for diet treatment. Within legume for the pea treatments SA and for the lupin treatments both SA and TA were significantly different at $P<0.05$. (Pea 100) denotes peas dry heat treated at 100°C for 10 minutes, (raw) denotes inclusion of legume with no treatment, (Lupin A5) and denotes autoclaved heat treatment of lupins at 120°C for 5 minutes.

7.5 Discussion:

The agglutination assay has provided some insight into the carbohydrate binding and or cell agglutination properties of the legumes tested.

7.5.1 Agglutination Experiments.

Of the raw legumes tested there was no significant difference seen in alkaline phosphatase (AP) in the two intestine sections except for vetch. A decision to use the combined sections 1 and 2 of the intestine was made and both dry and moist heats were used for treatments on each legume. The carbohydrate binding and agglutination activity of beans and vetch showed no great difference with heat treatment and were deemed unsuitable for further testing. Peas and lupins agglutination activity however showed significant responses to dry (peas) and autoclaved (lupins) heat treatments at specific time/temperature regimes and as such provided suitable medium for further testing.

Differences have been noted in abalone fed natural diets of algae as apposed to those fed manufactured diets where their digestive enzyme profiles changed or adapted to the diet over

time (Knauer *et al.*, 1996; Taylor, 1994). Given the digestive enzyme change there may also be a change in the glycosylation state of the apical membrane. The raw peas treatment shows a marked difference in the percentage of the control alkaline phosphatase activity (%CAP) in that the CRA were 20% less affected than the WRA suggesting a difference in the glycosylation state of the abalone due to rearing regime. Dry heat treatment of peas shows a curved effect for increasing temperature for both WRA and CRA that suggests that the assay may provide indications of optimal temperatures for reducing the carbohydrate binding and agglutination effects. The treatment of raw ingredients at temperatures of 150°C and 200°C are impractical in that protein quality can be impaired due to a maillard or browning reaction or cross linking of proteins rendering them indigestible by blocking sites of enzyme attack. Heat treatment of legumes also causes the formation of insoluble salts rendering phosphate limiting (Reddy *et al.*, 1982). There is a greater %CAP at 100°C in both WRA and CRA, however, in comparison to the raw treatment the WRA shows an improvement whereas the CRA shows no change. As 100°C is a more practical temperature to heat legumes for inclusion in diets it was decided that the final diet trial would use this temperature/ time regime. If the response observed was reflected in the diet effects we would expect to see only minor changes in enzyme profiles when commercially reared abalone are fed this diet.

The differences shown in agglutination effects on isolated apical membrane of WRA and CRA may also be attributable to the glycosylation pattern change with CRA as the autoclaved heat treatment of lupins shows a marked difference in response particularly at the lower time treatment. The heat treatment of 120°C is severe, however, there is obviously some change in components of lupins with 5 minutes heat that has markedly reduced the ability of the components to bind to the isolated apical membrane of WRA. The inclusion of the 20-minute 120°C treatment was to compare the results of a previous study (Chapter 4) in which a diet trial was performed with this heat treatment on lupins. In this previous feeding trial (Chapter 4 section 4.5.3.2) intestinal enzymes of the CRA showed the protease and carbohydrase activity to decrease with this heat treatment and the alkaline phosphatase (AP) activity to increase. Considering the current results of this temperature/time regime we see a decrease in %CAP with CRA, which indicates either carbohydrate binding, and/or cell agglutination. This may be the reason for the decreases in intestinal leucine amino-peptidase (LAP) and carbohydrate enzyme activity. The increase in AP activity in the previous study reflects what is seen here in that we see an inhibition of the AP enzyme by cell agglutination from the lupins, possibly reducing the availability of phosphorous. The abalone response is to increase the amount of enzyme protein in the intestine, providing increased AP activity, to release

phosphorous for uptake as was also seen in the earlier study. As the initial tests for carbohydrate binding and/or agglutination in this current study were on WRA intestinal apical membrane and the improvement seen was for autoclaved treatment no tests were done on the dry heat treatment of lupins on CRA intestinal apical membrane. The results of the CRA here suggest we would see a reduction in enzyme activity at 5 minutes heat treatment although not as to the extent of the 20-minute treatment. It was decided to use 5minute 120°C heat treatment of lupins for the feed trial.

7.5.2 Feeding Trial:

The results of the feeding trial have reflected the predicted outcomes proposed in that the heat-treated pea diet has shown a small positive effect on the intestinal enzyme function in that TA has significantly decreased or stayed the same suggesting less enzyme protein to gain the same SA. The heat-treated lupin has shown a small decrease in TA and hence enzyme protein however the SA in Sucrase, β-galactosidase and protease had decreased.

The heat treatment may have had an effect on antinutrient factors that could have been inhibiting the enzymes rather than a change in the agglutination property of components of peas. AP is significantly reduced in the PH group, which suggests that phosphorous is more available after heat treatment; therefore the abalone has a reduced requirement for AP activity. There was no change in the specific activity of maltase and sucrase, which means that the endogenous starch of peas has not been greatly affected by the dry heat. In a previous study (Chapter 4 section 4.5.3.2) increases in the activity of both maltase and sucrase where seen when peas were autoclaved and was most likely due to the starch being gelatinised creating more reducing ends and hence making it more digestible. Dry heat may not alter the endogenous starch to such an extent as the moist heat treatment. There is a possibility that the mucus cover of the intestine is reduced with the heat-treated peas as the β-galactosidase activity is significantly reduced. There was a relationship seen in an earlier study between high β-galactosidase activity and high mucus cover. The PH group has increased proportional SA:TA with all enzymes tested and suggests less inhibition of the enzymes by ANFs.

The heat treatment of lupins has provided a significant decrease in LAP in the LH group, which would be due to a maillard reaction with the high temperature (120°C) applied to the lupins. The decrease is however less than that seen in a previous study (Chapter 4 figure 4.6) where a 20 minute treatment of the 120°C temperature was applied. The interesting result is

that the TA has decreased in all enzymes tested which is similar to the previous study and a likely cause is a change in ANFs with the heat treatment. The extract from the legumes would contain soluble and insoluble components of which the insoluble, such as some non-starch polysaccharides, may have a greater impact on the intestinal enzyme function. The effect of autoclaving has possibly released some antinutrient factors that are heat labile that may also have an effect. This suggests that there is a hierarchy of effect from different antinutrient factors that will affect the abalones final digestive capacity.

7.6 Conclusion:

The agglutination assay appears to have been a reasonable predictor of the effects of a legume on the intestinal enzyme function in abalone in that the results of the assay relates to what was seen in the feeding trial. The agglutination pattern suggests there is a difference in the glycosylation pattern of the apical membrane between WRA and CRA as both react differently to raw and/or heat treatments. Further testing of lupins with dry heat treatments using intestine from CRA would be suggested. There may be value in testing at closer temperature ranges particularly in those areas, which would be more suitable for practical use in diets (ie less than 100°C) to find limits of heat treatment. This may be valuable in controlling processing temperature of diet by cooking or extruding to provide the optimum effect of denaturing ANFs and the reduction of carbohydrate binding or cell agglutination. Cost savings may apply to both farmers due to a better diet and to feed manufacturers in processing costs.

Chapter 8

The Effects of Legume based Diets on the Assimilation of Fatty Acids in the Abalone *Haliotis laevegata* Foot Tissue.

8. The Effects of Legume based Diets on the Assimilation of Fatty Acids in the Abalone *Haliotis laevegata* Foot Tissue.

8.1 Abstract:

Dietary polyunsaturated fatty acids (PUFA) provide important physiological functions in animals such as energy storage, precursors for prostaglandins in haemostasis, leucotrienes in inflammatory responses and cell membranes. Antinutrient factors in terrestrial plants can affect these functions.

The effect of feeding legumes on the assimilation of fatty acids in the foot muscle of abalone was investigated. Abalones were fed isonitrogenous diets using four legumes and soyflour as a protein source with treatments of raw, raw plus phytase and heat applied to the legume seeds and the soyflour. Wild reared abalones were used as a control.

The results indicate abalone have a poor capacity to chain elongate 18:2ω6 PUFA to 20:4ω6 PUFA. Low levels of the precursor 18:3ω3 PUFA in the tissue generally corresponds to a high level of longer chain PUFA in the tissue. Conversely high 18:3ω3 PUFA in the tissue corresponds to low levels of long chain PUFA. No significant trends can be seen with saturated fatty acids or monounsaturated fatty acids.

There are some indications of effects of the legume and/or its treatment on the assimilation of fatty acids in the foot muscle of abalone however the evidence here in this study is not conclusive. More work is needed to isolate antinutrient factors of legumes to see their effects on abalone fatty acid synthesis and assimilation. Diets may be produced to specifically alter the fatty acid profile of abalone and may be of use in meeting market preferences.

8.2 Introduction:

Lipids, in particular polyunsaturated fatty acids (PUFA) are important for a number of physiological functions in animals. These are as storage for energy, prostaglandin and leucotrienes precursors and in cell membranes. Important prostaglandins functions are in reproduction, haemostasis and the disease resistance of the abalone. If deficiencies are seen then reduced fecundity and an inability to repair damage to tissue, particularly the foot muscle in farmed situations, can often lead to opportunistic pathogenic attack. Leucotrienes function in inflammatory responses and if deficient can result in disease and can result in the eventual death of the animal. PUFA function in intestinal epithelial cell membrane is to maintain cell

structure and integrity and if compromised inefficiencies in enzyme and transport function may occur.

The naming of fatty acids has been simplified to that shown in figure 8.1 and will be used in this study. The systematic name for this fatty acid is, all cis-5,8,11,14,17-eicosapentenoic acid, the common name is Behenic acid. In the simplified system (20:5ω3), the first number refers to the number of carbon atoms, the second the number of double bonds and the third the position of the first double bond from the methyl (CH_3) end which is referred to as omega 3 (ω3).

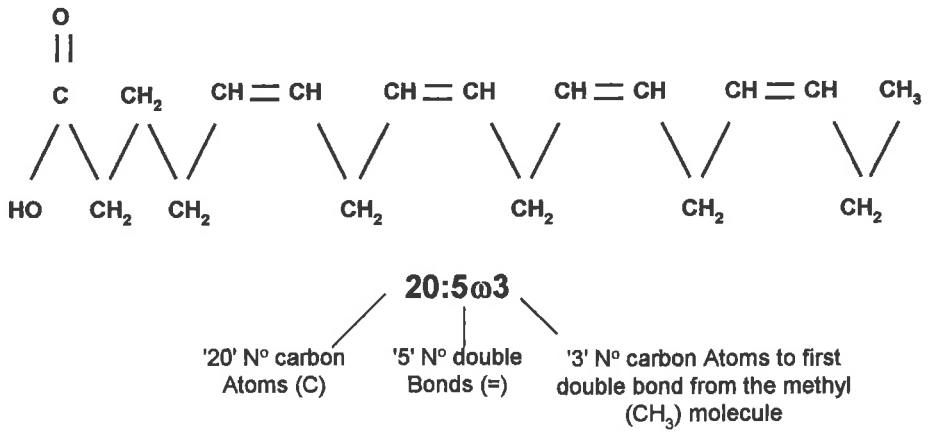


Figure 8.1: Structure of the Polyunsaturated Fatty Acid Eicosapentaenoic acid (20:5ω3).

Most marine animals cannot synthesise essential PUFA *de nova*. If insufficient amounts of these fatty acids are not supplied in the diet the following effects can be seen, low growth rates, reduced survival, low fecundity and lowered disease resistance (Kanazawa *et al.*, 1979; Sargent *et al.*, 1989; Uki *et al.*, 1986).

The nutritionally important PUFA are the ω6 and ω3 series (figure 8.2). These PUFA must be obtained from the animals diet. ω6 and ω3 PUFA are poorly, or unable to be, inter-converted by animals, however, animals can produce long chain 20 and 22 carbon PUFA from 18 chain PUFA. Most marine animals have a low efficiency in converting these PUFA therefore the animals have a specific "essential" dietary requirement especially 20:5ω3 and 22:6ω3. Studies on abalone using various seaweeds as diets suggest they are able to elongate short chain dietary ω3 series of fatty acids to long chain 20, 22 series (Dunstan *et al.*, 1996; Floreto *et al.*, 1996).

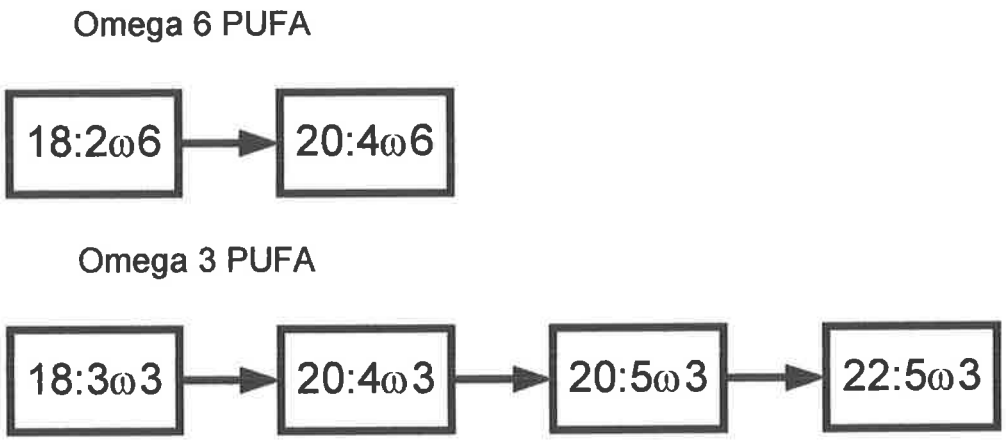


Figure 8.2: Chain Elongation and Desaturation of Short Chain 18 carbon Polyunsaturated Fatty Acids to long Chain 20 or 22 carbon Polyunsaturated Fatty Acids.

Abalone meat is generally low in lipid with most present in cellular membranes as polar lipids (De Koning, 1966). The viscera is the main lipid storage organ (Olley and Thrower, 1977). Abalone are different to most other marine species in that they have 20:5ω3 and 22:5ω3 as the main long chain PUFA in their tissue. Several studies have shown that enriching a diet with 20:4ω6 was less effective than 22:6ω3 whereas diets with both 20:5ω3 and 22:5ω3 produced higher growth rates (Dunstan *et al.*, 1996; Floreto *et al.*, 1996; Uki *et al.*, 1986; Van Barneveld *et al.*, 1998). The preferred diet of *Haliotis laevegata* is red algae which has high 20:5ω3 PUFA (17-32% of total lipids) and low 20:4ω6 (2-5%) and 18:2ω6 (1-7%) PUFA (Dunstan *et al.*, 2001; Foale and Day, 1992). Australian manufactured abalone diets use terrestrial plant sources such as soyflour and semolina, which contain high levels of 18:2ω6 PUFA. *Haliotis laevegata* fed manufactured diets have shown elevated levels of 18:2ω6 and 20:2ω6 (Dunstan *et al.*, 1996). In manufactured diets the abalone essential requirement for 20:5ω3 has come mainly from the inclusion of fish meal or fish oil (Fleming *et al.*, 1996). High inclusion levels of oil (4-9%) has been shown to have a detrimental effect on protein, carbohydrate and lipid digestibility (Dunstan *et al.*, 2001; Uki *et al.*, 1986; Van Barneveld *et al.*, 1998). Components of terrestrial plants such as pectin, lectins and other dietary fibres can effect bile acid excretion, sequester micellar components and effect the accumulation and absorption of lipid in the intestine (Vahouny *et al.*, 1980). In previous experiments the effect of various legumes on the structure and function of the intestine of abalone were seen. These effects may be a result of the integrity of the membrane and as such it would be important to understand possible changes in the fatty acid profile of the abalone with altered diets. Antinutrient factors (ANFs) in legumes may effect the digestion and conversion of PUFA to those essential for abalone growth and function.

In this study four legumes were used in diets, the field pea (*Pisum sativum* cv. Alma), yellow lupin (*Lupinus luteus*), faba bean (*Vicia faba* cv. Flord) and vetch (*Vicia sativa* cv. Blanchefleur) with treatments of raw, raw plus phytase for the release of phosphorous and heat (autoclaved) treatments to ameliorate anti-nutrient factors (ANFs). Soyflour, with similar treatments, was also used, as it is a major protein component in commercially manufactured abalone diets in Australia. Dry heat-treated peas and an additional autoclaved treatment of lupins were included. Wild reared abalone (WRA) were also examined and used as a control.

The aim was to examine the effects of feeding the above legumes, soyflour and treatments on the amounts and proportions of nutritionally important lipid components assimilated in the tissue of abalone.

8.3 Materials and Methods:

8.3.1 Animals

The abalones used in this experiment were from stocks held at South Australian Research and Development Institute (SARDI) West Beach. The abalone stocks held at SARDI were originally obtained from a commercial hatchery. Wild reared abalones (WRA) were collected from near Port Lincoln South Australia. Thirty-six animals were used for each treatment group.

8.3.2 Diets and Feeding.

Diets are shown in appendix A Table 1, Table 2,

Briefly fifteen isonitrogenous diets were used with legumes field peas (*Pisum sativum* cv Alma), lupins (*Lupinus luteus*), faba beans (*Vicia faba* cv Fjord) and vetch (*Vicia sativa* cv Blanchefleur) given three treatments, raw, raw plus enzyme (phytate), autoclaved (121^0C, 140kPa). A dry heat treatment of field peas (100°C for 10 minutes) was also included. Defatted soyflour was also formulated into diets with all three treatments applied.

Animals were fed to excess daily at 1700hrs for 14 days.

8.3.3 Aquarium System.

Abalone was housed in rectangular 10 litre acrylic aquarium. A flow through system was used with water filtered to a final 10μm nominal by composite sand filters. The average water

temperature maintained was 18^0C. Aquariums were cleaned by siphon daily. Six to ten animals were housed in each aquarium dependant on abalone size.

8.3.4 Abalone Tissue Sampling.

Abalone were killed between the hours of 0800 and 1100 with the foot tissue immediately snap frozen in liquid nitrogen and stored in liquid nitrogen for future analysis.

8.3.5 Lipid content and fatty acid analysis.

The procedures used were similar to those of Christie, (1989). An accurately weighed sample of approximately 2.0g of abalone tissue was homogenised in 30mL chloroform/methanol (2/1). The mixture was filtered under vacuum; the extract removed and the residue re-extracted with solvent. The combined extracts was reduced under vacuum then dried completely under a stream of nitrogen. The dried extract was weighed and recorded as the lipid content.

This residue was dissolved in 2mL of chloroform and various fatty acid fractions were separated by absorption chromatography using a small column of silica gel/florosil (30mm in a pasteur pipette). Triacylglycerols were eluted with 10mL chloroform, glycolipids with 10mL acetone and phospholipids with 10mL of methanol. The eluants were evaporated to dryness under a stream of nitrogen.

The phospholipid fraction was esterified under acid methylation conditions prior to fatty acid analysis by gas-liquid chromatography. The instrument used was a Hewlett Packard gas chromatograph (model 6890 II) using a BPX70 column with hydrogen as the carrier gas (6kPa head pressure) over a temperature range of 150-200°C ramped at 2°C per min. Individual fatty acids (C14-C22) were identified by comparison of retention times with those of authentic standards. Results were expressed per cent of total.

8.3.6 Chemicals.

All chemicals were the best available analytical grade and purchased from Sigma (Sydney Australia).

For simplicity in referencing abalone fed a particular protein source, the abalone intestine will be referred to after the protein source and or its treatment. For example the intestine of abalone fed the raw soyflour diet (S), raw soyflour + phytase (SP) or heated (autoclaved)

soyflour (SH). The intestine of abalone fed on a diet of peas with a dry heat treatment and abalone fed a diet of lupins with a 5 minute 120°C (autoclaved) heat treatment will be referenced as (PDH) and (L5H) respectively. Wild reared abalone will be referred to as WRA.

	Treatment		
Protein Source	Raw	Raw + Phytase	Heat
Soyflour	S	SP	SH
Lupin	L	LP	LH (20 min)
Vetch	V	VP	VH
Pea	P	PP	PH
Bean	B	BP	BH
Pea Dry Heat			PDH
Lupin			L5H (5 min)

8.4 Results:

The total percentage fatty acids recovered in the tissue of abalone ranged between 0.895 – 1.265% of the foot muscle. Of this total the majority was phospholipids with only ~3% being triacylglycerol. The main triacylglycerols were, 16:0, 18:0, 18:1ω9c, 18:1ω7c, 18:2ω6c and 20:1ω9. Due to the low content of triacylglycerol only phospholipids will be reported.

The major phospholipids found in the tissue analysis for all treatments are shown in Table 8.1. There are few trends seen in the saturated fatty acids (SFA) with treatment. With the addition of phytase to diets SP and PP tissue shows increases in both 16:0 and 18:0. LP, BP and VP tissue all show decreased amounts of 16:0 and 18:0. The abalone tissue monounsaturated fats (MUFA) levels show a similar trend for most treatments to the WRA in that 18:1ω9c accumulation is less than 18:1ω7c, exceptions to this are PP which is unchanged and BH tissue (highlighted with light shading) which shows 18:1ω7c lower. ω6 PUFA levels in abalone tissue are much higher in 18:2ω6c for all treatments in comparison to the WRA whereas the 20:4ω6 is much lower. The WRA tissue show relatively low levels of 18:3ω3 and high levels of the longer chain ω3 PUFA the highest being 22:5ω3. Abalone fed legume treatments show a particular general trend in that if the precursor 18:3ω3 levels are low (soy raw highlighted with medium shading) then the longer chain PUFA are high, conversely if the precursor is high (Pea H D10100 highlighted in medium shading) then the long chain PUFA are low. PP tissue shows no 22:5ω3 (highlighted with dark shading).

Table 8.1: Major Phospholipid Fatty Acids in the Foot Muscle of Wild Reared Abalone *Haliotis laevegata* and Abalone Fed Legume Based Diets with Various Treatments.

	SFA		MUFA		PUFA					
Diet	**16:0**	**18:0**	**18:1ω9c**	**18:1ω7c**	**18:2ω6c**	**20:4ω6**	**18:3ω3**	**20:4ω3**	**20:5ω3**	**22:5ω3**
Wild Reared	20.3	14.4	2.8	6.7	0.9	11.3	3.2	8.4	10.1	10.2
Soy Raw	22.6	10.7	2.6	4.4	4.4	4.1	**2.1**	**9.5**	**15.9**	**11.4**
Soy + Phytase	26.2	18.5	2.7	5.0	2.5	1.9	13.7	6.1	4.6	5.6
Soy H A20120	17.3	16.5	2.1	4.9	2.7	3.7	14.5	9.8	9.2	6.7
Lupin Raw	22.8	14.4	2.4	4.9	4.3	3.7	12.7	9.8	7.3	7.1
Lupin + Phytase	19.9	13.5	2.0	5.3	4.4	6.3	5.2	16.9	5.3	4.2
Lupin H A20120	17.7	13.5	2.3	5.1	4.0	3.7	9.1	10.6	12.4	9.0
Lupin H A5120	23.9	13.0	3.5	5.7	5.5	3.2	15.0	7.4	5.5	5.8
Pea Raw	23.4	14.3	2.8	4.0	3.7	2.1	17.0	5.9	6.4	6.5
Pea + Phytase	27.1	14.9	**5.2**	**5.1**	4.1	9.0	2.3	10.1	5.2	**0.0**
Pea H A20120	20.8	17.0	2.5	3.8	3.7	4.7	2.1	12.8	10.3	10.7
Pea H D10100	28.8	14.1	3.0	5.2	3.7	1.7	**17.7**	**6.0**	**3.3**	**3.2**
Bean Raw	21.9	15.6	2.7	5.3	3.7	3.0	15.0	6.6	8.1	3.0
Bean + Phytase	21.4	13.5	2.9	4.5	4.9	3.5	8.2	8.4	11.3	7.4
Bean H A20120	23.2	15.2	**7.6**	**3.6**	4.0	3.8	2.9	6.0	14.8	4.8
Vetch Raw	24.0	15.5	3.0	4.5	3.3	5.4	3.4	10.0	11.2	8.6
Vetch + Phytase	18.9	11.9	2.4	3.8	4.0	5.0	2.7	7.3	16.3	7.9
Vetch H A20120	27.8	19.3	3.7	4.9	3.3	2.7	12.4	7.2	5.2	1.7

Values are the mean only of n=3, SE was less than 4.6% of the mean in all cases. Pea + Phytase denotes a pea diet with an addition on phytase, pea H A20120 denotes peas heat treated by autoclaving for 20 minutes at 120°C prior to inclusion in the diet, Pea H D10100 denotes a pea diet with dry heat treatment of 10minutes at 100°C and Lupin H A5120 denotes lupins heat treated by autoclaved for 5 minutes at 120°C. Light shaded table cells show MUFA 18:ω9c levels higher than 18:1ω7c. Medium shaded table cells shows opposite effects of legume source and treatment on PUFA levels. Dark shaded table cell shows no PUFA 22:5ω3 in abalone muscle fed the pea + phytase diet.

The major phospholipid pathways show there is between 80 and 89% of all phospholipids accounted for in these pathways, minor intermediates are not shown (Table 8.2). The trend seen is that if one pathway shows high levels of fatty acid then there is a reduction of either one or both of the other pathways such that the overall level remains little changed. SP tissue has a high 16:0 pathway levels (highlighted with light shading) in comparison to the WRA a

much lower 18:2ω6 pathway and similar 18:3ω3 pathway. PP tissue has a much higher 16:0 pathway (highlighted with light shading) to the WRA and similar to SP however the 18:2ω6 pathway is higher than the WRA and the 18:3ω3 pathway much lower. The overall total phospholipids are only reduced 3% compared to P tissue. LH tissue on the other hand shows low 16:0 pathway compared to WRA and the other lupin treatments and a much higher 18:3ω3 pathway. The overall total phospholipids are reduced by 2% compared to L tissue. With the exception of BP tissue (highlighted with dark shading) all other abalone fed legumes with a phytase addition show a decrease in the overall total phospholipids accounted for.

Table 8.2: Major Phospholipid Fatty acid Pathway Levels (% of total Phospholipids) for 16:0 and Dietary Acquired 18:2ω6 and 18:3ω3 Precursor Polyunsaturated Fatty Acids in the Foot Tissue of Wild Reared Abalone *Haliotis laevegata* and Abalone Fed Legume Based Diets with Various Treatments.

	16:0 Pathway	18:2ω6 Pathway	18:3ω3 Pathway	% Total Phospholipids
Wild Reared	44.2	12.2	31.9	88.2
Soy Raw	40.4	8.5	39.0	87.8
Soy + Phytase	52.3	4.4	30.1	86.7
Soy H A20120	40.8	6.4	40.2	87.4
Lupin Raw	44.5	7.9	36.8	89.2
Lupin + Phytase	40.8	10.6	31.6	83.1
Lupin H A20120	38.6	7.6	41.1	87.3
Lupin H A5120	46.1	8.7	33.7	88.5
Pea Raw	44.4	5.8	35.8	86.0
Pea + Phytase	52.3	13.1	17.5	83.0
Pea H A20120	44.1	8.4	35.8	88.4
Pea H D10100	51.0	5.4	30.3	86.7
Bean Raw	45.4	6.7	32.9	85.0
Bean + Phytase	42.3	8.4	35.3	86.0
Bean H A20120	49.5	7.8	28.5	85.8
Vetch Raw	47.0	8.7	33.2	88.9
Vetch + Phytase	36.9	9.0	34.2	80.2
Vetch H A20120	55.7	6.0	26.5	88.2

Pea + Phytase denotes a pea diet with an addition on phytase, pea H A20120 denotes peas heat treated by autoclaving for 20 minutes at 120°C prior to inclusion in the diet, Pea H D10100 denotes a pea diet with dry heat treatment of 10minutes at 100°C and Lupin H A5120 denotes lupins heat treated by autoclaved for 5 minutes at 120°C. Light shaded table cells show high levels of 16:0 pathway relates to lower levels in 18:2ω6 and 18:3ω3 pathways. Dark shaded table calls show all raw + phytase treatments with the exception of beans have lower total phospholipids.

8.5 Discussion:

The WRA tissue shows consistent results to other researchers (Dunstan *et al.*, 2001) who have tested *Haliotis laevegata* foot tissue and this tissue reflects the composition of the preferred red algae diet. The red algae have high levels of the essential ω3 PUFA in particular 20:5ω3 and the low levels of 18:2ω6 and 20:4ω6. The main ω6 PUFA in WRA tissue is 20:4ω6 which indicates either the WRA chain elongates 18:2ω6 almost completely or it is very efficient at incorporating the low levels of 20:4ω6 found in red algae into the tissue. Dunstan *et al.* (2001) found that abalone fed diets with high levels of the precursors 18:2ω6 and 18:3ω3 have low growth rates compared to those fed diets supplemented with the longer chained 20:4ω6 and 20:5ω3 PUFA and suggested the abalone have limited capacity to chain elongate these precursors.

With the ω3 PUFA, WRA chain elongates 20:5ω3 to 22:5ω3 as there is only trace amounts of 22:5ω3 in red algae (Dunstan *et al.*, 2001; Johns *et al.*, 1979). The low capability to chain elongate 18:2ω6 is reflected in the tissue of abalone fed the raw and treated legume diets as the level of 18:2ω6 fatty acids are high in all these terrestrial plants and the levels of 20:4ω6 low. However, no abalone fed treatments of legumes shows as high a value of 20:4ω6 PUFA as the WRA and is likely due to natural red algae diet which is much higher in 20:4ω6 than 18:2ω6 PUFA (Dunstan *et al.*, 2001). The 18:3ω3 pathway has quite different results within each legume and treatment and between legumes. Pea is the only legume with significant amounts of 18:3ω3 PUFA (Petterson and MacIntosh, 1994) and P tissue has the highest level of 18:3ω3 PUFA. Jack mackerel oil was used in these diets and has high 20:5ω3 PUFA (10%) (Dunstan *et al.*, 2001) however this is not reflected in the long chain PUFA amounts seen in the P group tissue. P group tissue has medium levels of 20:5ω3 and 22:5ω3 PUFA that may mean that the abalone has enough long chain PUFA or the precursors to elongate for its lipid requirements. A feedback mechanism may be in place in this situation to regulate enzymes that elongate fatty acids. Alternatively components of peas may be inhibiting the enzymes that provide the elongation and may be the reason for the medium levels seen. The PP group tissue may be a good example of enzyme inhibition as this diet produced negative effects on structure and function of the intestine in a previous study (Chapter 4). The 18:3ω3 PUFA level in PP tissue is much reduced from the P tissue however the following long chain 20:4ω3 is higher than P indicating that a chain elongation has occurred. With the longer chain PUFA this is not the case as there was no 22:5ω3 detected in the PP group suggesting the enzymes

that chain elongate at this stage are either not functional or not present. There is also the possibility that some component of peas released by the phytase has stopped the assimilation of these long chain PUFA into the tissue. In a previous experiment (Chapter 4) it was also noted that diets of raw soyflour and raw vetch the intestine of abalone fed these diets both had a dense mucus cover. They also had tall villus structure indicating cell proliferation and this may be reflected in this fatty acid analysis. The S and V groups' tissue have only low levels of 18:3ω3 PUFA however the following long chain PUFA is at a much higher level or comparable to WRA tissue. In this case there is no inhibition of the chain elongating enzymes and the cell turnover may require more of these essential PUFA. The heat treatment of soyflour has effectively reversed the pattern of PUFA assimilation in the abalone tissue however there are still high levels of 20:5ω3 PUFA.

The SFA and MUFA component of the abalone tissue varies with diet and treatment however no specific trends are seen other than if the level of these fatty acids are high then there is a concurrent decrease in the dietary PUFA assimilated into the tissue.

The lipid composition in all cases has been changed with diet and may have ramifications in the flavour and odour of abalone meat. Should we loose the long chain PUFA balance from the tissue it may loose the seafood or "fishy' odour and may lead to changes in the foot muscle texture. The flavours associated with wild abalone may be more in demand in the market place, however, from these results it may be possible to change the fatty acid balance and hence flavour of the foot muscle to suit market requirements.

8.6 Conclusion:

There is no conclusive evidence in this study that suggests anti nutrient factors in legumes are having an effect on fatty acid synthesis or assimilation into the abalone foot tissue. If there are any effects they are most likely confounded by the inclusion of the jack mackerel oil. More work is required to isolate the effects of the legume themselves and may be achieved by reformulating the diets with varying inclusion levels of oil and legume. There is strong evidence that diets may be formulated to alter the flavour of the foot muscle and may help in the marketing and price obtained for the final product.

Chapter 9

General Discussion

9 General Discussion:

9.1 Abstract:

There are conflicting accounts as to the function of the intestine of abalone. The intestine has long been considered to be an area for organising faecal rods (Campbell, 1965) while some researchers believe it may provide a digestive and absorptive function although not to the same degree as earlier sections of the gut (Bevelander, 1988; Harris *et al.*, 1998). To understand nutrient uptake in abalone this area needed to be resolved as to what capacity the abalone intestine has in the final digestion of food and subsequent uptake.

The use of terrestrial plant-derived materials such as legume seeds and oil seeds as abalone feed is limited by the presence of a wide variety of antinutritional substances. Important among these are protease inhibitors, phytates, tannins, lectins, oligosaccharides and non-starch polysaccharides, alkaloids and antigenic compounds. Little agreement exists between the results of different studies on other marine and terrestrial species as to the specific effects of antinutrients. Most studies have been conducted using an ingredient rich in one particular factor and the observed effects have been attributed to this factor without considering other antinutrients present in the ingredient or interactions between them. The effects of these substances on abalone intestinal structure and function have been the focus of this study. The effectiveness of common processing techniques such as dry and moist heating and enzyme treatment in ameliorating the deleterious effects of antinutrients from feed materials was also studied.

Tentatively, protease and carbohydrase inhibitors, phytates, antigenic compounds, legume inclusion level, starch type and starch inclusion level, carbohydrate binding and agglutination and inert filler inclusion are likely to affect abalone intestinal morphology and function resulting in reduced digestive performance. There appears to be a hierarchy of effect from different ANFs that will affect the abalone final digestive capacity.

More insights into the nutritional, physiological and ecological effects of antinutrients on abalone intestinal structure and function and farming systems need to be accumulated through studies using purified individual antinutrients and their mixes in proportions similar to those in alternative nutritional sources in abalone feeds. Such studies would provide data useful for designing optimum inclusion levels of terrestrial plant-derived materials and treatment methods that would ameliorate the negative effects of antinutrient factors (ANFs).

9.2 Introduction:

Feed is the largest operating cost in intensive aquaculture. Protein is essential for soft tissue growth and is the most expensive component in artificial diets. Fishmeal is used extensively in many aquaculture diets as it supplies protein, energy and essential fatty acids (Lovell, 1988). Aquaculture has become the fastest growing food production sector in the world with an average annual increase of around 10% since 1984 as compared to 3% for livestock meat and a 1.6% increase in capture fisheries (Francis *et al.*, 2001). To sustain high rates of increase in aquaculture production a matching increase in the levels of production of fishmeal is required. The very rapid increase in aquaculture particularly in Asia means competition for limited global supplies of fishmeal is likely (Naylor *et al.*, 2000). The need for alternative protein sources to replace fishmeal in aquaculture was strongly recommended by the Second International Symposium on Sustainable Aquaculture (1998) (Naylor *et al.*, 2000). In Australia the current manufactured abalone diets use fishmeal and soyflour as their main protein sources, the bulk of which is imported at high cost. Current research in Australia is looking at locally grown legumes as potential replacements for fishmeal and soyflour. The effect of antinutrient factors (ANFs) in legumes on the structure and function of the gastrointestinal tract have caused problems in their use in many fish and terrestrial animals and needs to be considered when formulating legumes into abalone diets.

9.3 Antinutrient factors.

Most alternative terrestrial plant-derived nutrient sources, including legumes, are known to contain a wide variety of ANFs. ANFs, which either by themselves, or through their metabolic products, interfere with food utilisation and affect the health and production of animals. The main effects seen are factors which affect protein utilisation such as protease inhibitors, tannins and lectins, factors affecting mineral utilisation including phytates and other effects of miscellaneous factors such as mycotoxins, cyanogens alkaloids phytoestrogens and saponins (Smits and Annison, 1996). A commonly employed treatment for destroying ANFs is with heat however some antinutrients can withstand thermal processing. Heat labile factors include protease inhibitors, phytates, lectins, whereas heat stable factors are saponins, non-starch polysaccharides, antigenic proteins and estrogens (Rumsey *et al.*, 1993).

A review of the effects of ANFs in fish has been presented by Francis *et al.*, (2001) of which growth performance was the major measure reported. Only 4 of the 56 studies reviewed

reported intestinal epithelial morphology, one from the effects of phytate and three from the effects of saponins, with all showing abnormalities and damage to the intestine. In the four studies mentioned growth performance was also reported. In general, in all these studies, the effects of ANFs reported on growth performance, which was either, a depression or no change from the control diets used. While growth is still achievable using terrestrial plant-derived nutrient resources, optimizing this growth and health considerations are important if production systems are to be profitable.

The endeavor of this study was to provide information on the nature, possible mode of action and effects of ANFs on the structure and function of the intestine of abalone and known methods to eliminate the effects of potent ANFs found in terrestrial plant sources. It was hoped that the information derived from this study would enable a better understanding of the effects and utilisation of terrestrial plant resources of protein and carbohydrate for abalone aquaculture production.

9.4 Intestinal Morphology.

The intestine of the abalone is rarely considered in any study on the morphology or function that it provides. There has been three comprehensive studies on the morphology of the gastrointestinal tract (GIT) of abalone of which all have provided little information on the morphology of the intestine (Bevelander, 1988; Campbell, 1965; Harris *et al.*, 1998). The intestine is described as having 5 sections and the most notable features mentioned in these studies above are the typhlosole, the cilia on the apical membrane and the spherules that cover this area. Harris *et al.* (1998) has been the only group to look at the gastrointestinal tract in relation to diet and in their case, feed restriction. Here in this study in both wild (WRA) and commercially (CRA) reared abalone the typhlosole, spherules and cilia were the predominant features, however, the intestine has three distinct areas when physical morphology at the lumen is considered. There is a section immediately after the stomach, referred to as section 1 in this current study, which has a prominent typhlosole with approximately 11 other single ridges. This area abruptly ends leaving only the typhlosole as the main feature for what has been termed section 2 in this study and the anal or rectum region follows this.

In chapter 3 the first negative effects from the use of terrestrial plants were seen in the intestine of the CRA groups fed manufactured abalone diets with soyflour and fishmeal as

their protein sources. There were also differences between the two CRA groups tested with feed sourced from different manufacturers. The predominant external morphological change seen in the CRA, in comparison to the WRA, was the increase in the mucus cover over the epithelium, which produced a many-layered cover such that the apical membrane features (spherules) were not seen. At the cellular level there was an increase in secretory cells, the cell nuclei were more centrally located, non vacuolated cells increased in number and there was a profound infiltration of haemocytes in the lamina propria and interspersed among the columnar cells. The response seen is typical of an inflammatory response and is most likely due to ANFs particularly antigenic proteins.

The same response was seen in the abalone fed the raw legume diets and the treated legumes. Heat treatment of legumes and soyflour has made little difference to the number of haemocytes seen in the tissue which suggests that heat stable ANFs are responsible, possibly antigenic proteins (Rumsey *et al.*, 1993). The effects of other ANFs cannot be discounted and may have been partially responsible for morphological changes. The moist heat treatment of legumes (autoclaved 20 minutes at 120°C 130kpa) has likely caused a maillard reaction resulting in protein being bound that renders it indigestible. The effects are seen in the intestine of abalone fed the heat-treated legume and soyflour diets with the villus structure showing a reduction in height and crypt cell proliferation. Low protein diets are known to alter the morphology of the intestine with decreased villus height, crypt cell proliferation rates, thinning of the intestinal wall and increased basal width of the villus commonly noticed (King *et al.*, 1983; Smith and Peacock, 1989; Syme, 1982; Syme and Smith, 1982). In abalone fed the heated vetch diet the density of the mucus cover of the epithelium was greatly reduced, as was the number of secretory cells indicating that this treatment has had a positive effect probably by reducing ANFs other than antigenic proteins.

Phytate can cause hypertrophy of the intestine, cytoplasmic vacuolization and also intestinal cataracts in fish (Richardson *et al.*, 1985; Sugiera *et al.*, 1999). The addition of phytase to the legume diets in this current study showed various effects on the morphology of the abalone intestine. This ranged from little difference, in comparison to the abalone fed the raw diets, with lupins, vetch and bean diets, a more even structure to the villus in abalone fed soyflour to a disruption of the villus structure when abalone were fed pea diets. The difference in the intestine of abalone fed the pea diet may be the release of cystine, which has an inhibitory effect on alkaline phosphatase enzyme, which releases phosphorous. There may not be sufficient phosphorous in this case for normal cellular functions and in particular for use in

membrane structure. In the intestine of abalone fed the soyflour a reduction in phytic acid and a release of phosphorous may be the reason for the improvement in morphology. The abalone fed the raw pea diet had a similar intestinal morphology to the WRA and is possibly due to the fact that peas have less ANFs than the other legumes tested (Petterson and MacIntosh, 1994). A more notable change in the morphology was seen in section 1 of the intestine from abalone fed all lupin treatments that showed few spherules. It is not known what function the spherules have however any dietary inclusion that removes these structures would have to be considered with caution.

Soyflour and legumes when included in animal diets have shown to cause adverse effects on the morphology of the intestine. The changes have been ascribed to the presence of ANFs particularly protease inhibitors and allergenic proteins and lectins in soyflour, (Huisman and van der Poel, 1988; Liener, 1994), the cyanoalanine compounds common in vetch (Ressler *et al.*, 1968) and tannins in beans (Marquardt and Ward, 1979). The differences seen here in this study may reflect the particular ANF composition of the seeds.

9.5 Functional Characteristics.

There are few studies on the function of the intestine of abalone and it is only recently that Serviere-Zaragoza *et al.*, (1997) found the highest proteolytic activity region to be in the intestine, with chymotrypsin 10 times higher than trypsin. This indicates a proactive role for the intestine in the digestion of feed. Given this result it could be said that final carbohydrate digestion could also be found in the abalone intestine.

The apical membrane of the intestine was isolated by a magnesium precipitation technique. When tested for marker enzymes of apical, intracellular and basolateral membranes this showed that the isolation procedure provided results comparable to other mammalian and fish studies and would be suitable for testing intestinal enzyme function in the abalone.

The initial study in chapter 3 on WRA and CRA showed final digestive enzymes in the apical membrane of the intestine (alkaline phosphatase, maltase, sucrase, β-galactosidase and leucine amino peptidase) that are common in most mammals and fish are seen in abalone. Of the carbohydrases tested (maltase, sucrase and β-galactosidase) maltase had the greatest activity which is similar to that found by Bennett *et al.* (1971) and Clark and Jowett, (1978) although their tests were on other sections or the entire gastrointestinal tract, not the isolated

intestine. Maltase is often found to have the greatest activity of the disaccharides in the intestine of mammals and aquatic species (Bamba *et al.*, 1990; Ciancio and Chang, 1992; Clark *et al.*, 1984; Glass *et al.*, 1987; Harrison and Webster, 1971; Krogdahl *et al.*, 1999). There was an indication that β-galactosidase was associated with mucus levels and may be used as an indicator of dietary components that may induce intestinal secretions.

In abalone groups fed the manufactured, raw soyflour and legume diets the intestinal enzymes activities studied suggested components of these diets might be inhibiting these enzymes. The total activity of the intestinal enzymes was higher than that found in the intestine of WRA. Several factors may contribute to this result; there may have been lectin binding and agglutination of the apical membrane, the dense cover of mucus may interfere with enzymes interfacing with the substrate or a direct inhibition of the enzymes by competitive and non-competitive inhibition by components of the diet. The bio availability of essential nutrients can also account for changes in enzyme activity as minerals such as zinc are linked to peptidases and alkaline phosphatase and are needed for activation of these enzymes (Yamamoto *et al.*, 1991). All the effects can be attributable to ANFs as these effects are seen in other animals and fish using these protein sources in diets (Francis *et al.*, 2001; Irish *et al.*, 1999; Mizuno *et al.*, 1992). Many of these effects can be ameliorated by treatment of the seed by heat or enzyme supplementation. Several other possibilities were suggested for differences in enzyme activity and were related to the type of starch in the diet and the inclusion levels of the legume in the diets. The heat and enzyme treatments, starch type, inert filler, and legume inclusion levels were tested in this study.

9.5.1 Heat Treatment.

The heat treatment of soyflour and the legumes has most likely resulted in a maillard reaction and this was seen in the reduction in protease activity. Protein can become indigestible due to this reaction as well as lysine becoming limiting due to it being complexed with sugars. In this study the levels of protease activity decreased in all groups. The moist heat treatment of peas and vetch however caused a significant increase in maltase activity due to the endogenous starch of these seeds being gelatinized. This change in starch structure and the increased carbohydrase activity suggests the abalone need to have a starch that is easily digested by these enzymes.

9.5.2 Enzyme Treatment.

The addition of phytase to the diets has probably made phosphorous more available and also other micro-nutrients which may be significant as phosphorous is used in cellular metabolism ultimately affecting growth and health status. The only protein source to have a negative effect on the intestinal enzymes of abalone was from the use of phytase with peas. A significant increase in the digestibility of cysteine was found in a concurrent study by Vanderpeer *et al.* (1999) using the same diet and as cysteine is an inhibitor of alkaline phosphatase the effects of reduced availability of phosphorous is seen not only on the intestinal enzyme function but also on the morphology as discussed above. The addition of phytase needs to be assessed on an individual basis however it appears that abalone when fed legumes with high phytate content do benefit from this treatment.

9.5.3 Starch Type.

Numerous carbohydrate hydrolytic enzymes are found in the gastrointestinal tract of abalone the presence of which can be related to the structural polysaccharide linkages found in natural diets (Clark and Jowett, 1978; Knauer *et al.*, 1996; McLean, 1970; Nakada and Sweeny, 1967). It has been considered by researchers that these enzymes would allow the use of terrestrial plants to be used in abalone diets (Fleming *et al.*, 1996). The starch found in preferred red algae consumed by *Haliotis laevegata* is a floridean starch similar in structure to amylopectin and hence has many non-reducing ends (Craigie, 1974). In this study abalone do not appear to have a great capacity to digest the storage starches in legumes (amylose) as changes to higher amylopectin levels provide greater carbohydrase activity. When moist heat treated (pregelled) starch is included in diets carbohydrate activity increases, however at high levels there is a decrease in activity which is in agreement with other research on aquatic species (Hemre *et al.*, 1979; Henrichfreise and Pfeffer, 1992; Pfeffer *et al.*, 1991).

9.5.4 Inclusion Level of Legumes.

The protein weight content of legumes varies from high levels found in lupins (27-40%) to low levels in peas (19-27%) which requires inclusion levels of low protein legumes to be high to provide isonitrogenous diets. In aquatic species high inclusion levels of legumes and soyflour have decreased protein and energy digestibility (Arndt *et al.*, 1999; Pfeffer *et al.*, 1995). The high levels of peas, beans and vetch to maintain diets isonitrogenous were shown

to effect intestinal enzyme activity as a reduction in inclusion levels increased enzyme activity. The reduced inclusion would also mean a reduction in the ANFs present in the diet.

9.5.5 Inert Filler Inclusion.

The inclusion of bentonite and in particular kaolin have provided a stimulatory effect on the intestinal enzymes of abalone with a 10% inclusion of kaolin showing the greatest stimulation of enzyme activity as well as a greater reduction in the total activity. The reduction in total activity suggests that not as much enzyme protein is required to achieve digestion as with the base diet. The effects of inert filler inclusion need considerable more study in abalone however there are not only beneficial effects to be gained from diet improvement but also in water quality in farming situations.

9.5.6 Carbohydrate Binding and Cell Agglutination.

The assay used in this study is a modified version of (Irish *et al.*, 1999) designed to test the entire legume seed rather than isolated components of the legume. The agglutination pattern suggests that diet or rearing regime has an effect on the glycosylation pattern of the cell membrane. The feeding trial following a selection of the diets based on this agglutination pattern proved to correlate well with the predicted results. Further studies would be required to confirm the preliminary results achieved in this thesis. The ability to test raw and treated feed sources without the need to do lengthy feed and growth trials may be beneficial and cost saving to the industry.

9.5.7 Fatty Acid Profile, Dietary Effects.

The fatty acid profile of the WRA foot muscle in this study showed agreement with other researchers (Dunstan *et al.*, 2001). No direct evidence that ANFs were having an effect on fatty acid chain elongation and desaturation was seen however this preliminary study does point to certain polyunsaturated fatty acids (PUFA) that need to be supplemented to diets formulated with terrestrial plant sources. These are 20:4ω6 and 20:5ω3 which is in agreement with (Dunstan *et al.*, 2001). The lipid composition was changed in all cases with legume diets and treatments and may affect the flavour of the meat. The markets may not respond well to flesh that looses the fishy taste often associated with long chain fatty acids.

9.5.8 Environmental Effects.

The inhibitory effects seen in this study on enzyme activity by ANFs would mean that a percentage of the diet would not be fully digested and past out of the abalone in the faeces into the tanks and water reticulation systems of the farm. A number of the treatments used in this current study have reduced the total activity of the enzymes, corresponding to a reduced amount of enzyme protein in the intestine. This result indicates that deleterious effects of the ANFs have been reduced and a more 'efficient' enzyme activity can be implied. This more efficient activity may result in less undigested feed entering the farm system improving water quality and possibly reducing the amount of feed required for optimum growth. In particular, in aquaculture the properties of non-nutritive fillers, such as ion-exchange and absorption, have been exploited to make more efficient use of nitrogen in nutrition to control moisture and ammonia content of faeces, to purify recirculating hatchery waters and to reduce the nitrogen content of hatchery waste water (Mumpton and Fishman, 1977). These properties would be of benefit to abalone farms even if no improvement were found on intestinal function providing however, that no adverse effects of the filler are seen.

9.6 Conclusions and Future Work.

In this study the terrestrial plant sources used, soyflour and legumes, contain more than one antinutritional substance and it is therefore difficult to isolate one particular substance as the causative agent for the negative effects seen on abalone intestinal morphology and function. The use of legumes in abalone diets could produce adverse effects and decrease productivity however more information is required on particular individual ANFs and there interactions with each other before making conclusions as to their full potential in abalone diets. The grow out period for abalone is long in comparison to other animals and fish, as such the diets need to be of optimal quality so that no adverse effects effect the production cycle and health of the abalone. Tentatively, protease and carbohydrase inhibitors, phytates, antigenic compounds, legume inclusion level, starch type and starch inclusion level, carbohydrate binding and agglutination and inert filler inclusion are likely to affect abalone intestinal morphology and function resulting in reduced performance. The common processing techniques of heat treatment and enzyme supplementation appear to have provided some success however caution needs to be exercised as the phytase treatment of peas has had an adverse effect on the enzyme activity. There appears to be a hierarchy of effect from different ANFs that will affect the abalone final digestive capacity. There are similarities with other

animals and fish as to the effects of ANFs seen in abalone and as such the plethora of research studies on ANFs should be used to expedite research on abalone diets.

The present research is one of the first to comprehensively study the role of the abalone intestine with regard to the effects of diet. The research has followed the current trend in seeking cheaper alternative protein sources for use in manufactured abalone diets. Some of the results of this study would be regarded as preliminary and therefore not conclusive. It is hoped that the current research would open avenues of discussion and more concerted investigations into the areas covered, more specifically the following would need further investigation:

1 The role of the spherules needs to be established as they may be a primitive form of microvillus and have a similar role.

2 More insights into the nutritional, physiological and ecological effects of antinutrients on abalone intestinal structure and function and farming systems need to be accumulated through studies using purified individual antinutrients and their mixes in proportions similar to those that would be found in alternative nutritional sources in abalone feeds. Such studies would provide data useful for designing optimum inclusion levels of terrestrial plant-derived materials and treatment methods that would ameliorate the negative effects of antinutrient factors (ANFs).

3 There is little information on the mechanisms of nutrient uptake in abalone. The identification of nutrient transporters and the effects of ANFs on their activity would be beneficial in providing diets that are optimal for abalone.

4 The techniques used in this study namely the isolation of the apical membrane and the agglutination assay may find wider application not only in the abalone industry but also in other intensive agricultural farmed species. If rapid assessment of dietary components and treatment of these components can be made prior to inclusion in research diets, considerable time and cost savings may be made.

5 This method of study should be used concurrently with digestibility studies such that a more complete understanding of the proposed nutrient source and possible deleterious effects not found in digestibility studies can also be assessed and considered before nutrient sources are formulation into diets.

Appendix A

Diets

It must be noted that the diets used in this study were also used by M. Vanderpeer (SARDI Aquatic Science Adelaide S.A. Australia) in a concurrent study on the digestibility of legumes and is the reason for the addition of chromic oxide. Chromic oxide is an indigestible marker that is detected and used for subsequent digestibility calculations.

Legume diets.

The following table (Table A1) shows the base ingredients, which are unchanged in each diet. The protein source, kaolin and pregelled starch levels plus any other additions are shown in table 2.

Table A1: Unchanged Base Ingredients in Legume Diets (g/kg, air dry basis).

Jack Mackerel Oil	20
Mineral Premix [1]	2
Vitamin Premix [1]	3
Vitamin C [2]	0.5
Vitamin E [3]	0.1
Sodium Alginate	5
Chromic Oxide	5

1. Vitamin and mineral premixes as described by Uki *et al* (1985).
2. Ascorbic Acid.
3. DL-alpha tocopheryl acetate.

Table A2: Legume Inclusion and Other Variable Ingredients (g/kg, air dry basis).

Raw and Heat Treated (Autoclaved and Dry Heat)

Soyflour [4]	333.3				
Lupins [5]		389.6			
Vetch [6]			638.3		
Peas [7]				742.6	
Beans [8]					663.7
Kaolin	200	200	200	200	200
PGS [9]	431.1	374.8	126.1	21.8	100.7

Raw + Phytase

Soyflour [4]	333.3				
Lupins [5]		389.6			
Vetch [6]			638.3		
Peas [7]				742.6	
Beans [8]					663.7
Kaolin	200	200	200	200	200
PGS [9]	430.9	374.6	125.9	21.6	100.5
Phytase [10]	0.2	0.2	0.2	0.2	0.2

4. Defatted Soyflour (Bakers Nutrisoy; Ace Chemicals, Adelaide, SA Australia).
5. *Lupinus luteus.*
6. *Vicia sativa.*
7. *Pisum sativum.*
8. *Vicia faba.*
9. Pregelled Waxy Maize Starch.
10. Natuphos ®5000G, BASF Australia Ltd, Auburn, NSW, Australia.

Table A3: Legume with Reduced Pregelled Starch and Reduced Inclusion Level of Legume Compositions (g/kg, air dry basis).

	Reduced PGS		Reduced Inclusion Levels		
Soyflour [4]	333.3		129.7	158.3	137.5
Lupins [4]		389.6			
Vetch [6]			390		
Peas [7]				390	
Beans [8]					390
Kaolin	532.3	476	200	200	200
PGS [9]	100	100	254.7	226.1	246.9

Starch Diets:

Table A4: Starch Base Diet and Reduced Pregelled Starch Composition (PGS)(g/kg, air dry basis).

	PGS	Reduced PGS
PGS	989.4	489.4
Kaolin	0	500
Vitamin Mix	0.5	0.5
Mineral Mix	0.1	0.1
Vitamin C	3	3
Vitamin E	2	2
Chromic Oxide	5	5

Inert Filler Diets:

Table A5: Inert Filler Diet Composition (g/kg, air dry basis).

Pregelled Starch		50							
Bentonite			50						
Diatomacious Earth				50					
Sand					50				
Kaolin 5%						50			
Kaolin 10%							100		
Kaolin 15%								150	
Kaolin 20%									200
Semolina	495	445	445	445	445	445	395	345	295
Soyflour	360	360	360	360	360	360	360	360	360
Fishmeal	80	80	80	80	80	80	80	80	80
Casein	20	20	20	20	20	20	20	20	20
Jack Mackerel Oil	20	20	20	20	20	20	20	20	20
Sodium Phosphate	5	5	5	5	5	5	5	5	5
Mineral Premix	4	4	4	4	4	4	4	4	4
Vitamin Premix	2	2	2	2	2	2	2	2	2
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin E	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sodium Alginate	8	8	8	8	8	8	8	8	8
Chromic Oxide	5	5	5	5	5	5	5	5	5

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