Effects of soybean meal and water temperature on the mucus layer and the development of sub-acute enteritis in Yellowtail Kingfish (*Seriola lalandi*)

By

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Declaration

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

Matthew Bansemer

30th May 2011

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PART A: LITERATURE REVIEW

Introduction

The world‘s fish stocks are fully, or over exploited, with an increase in harvest from the wild fishery not possible (Hardy, 1999). In order to supply the populations increased demand for fish a greater reliance on aquaculture is anticipated. At present, aquaculture feeds contain a high proportion of fish meal and fish oil. In the next decade the demand for fish meal is likely to exceed supply, highlighting the importance of protein alternatives being established for the expansion of aquaculture (Gatlin *et al.*, 2007).

Plant proteins from soybean, wheat, corn, lupins and canola have been identified as possible key alternatives to fish meal due to their cheap price, availability and sustainability (Merrifield *et al.*, 2009). However, a number of implications arise from increasing levels of plant-derived alternatives in feeds including deficiencies in certain amino acids, low palatability and antinutritional factors (Hardy, 1996).

The quantity of plant feed inclusion in aquafeeds is species-specific and related to the physiological adaptations of individual species (Gatlin *et al.*, 2007). A dietary inclusion of less than 10% soybean meal for Atlantic salmon induced intestinal inflammation described as sub-acute enteritis of the distal epithelial mucosa (Baeverfjord and Krogdahl, 1996).

The mucus layer is the first line of defence for the epithelium of the gastrointestinal tract against detrimental compounds and pathogenic bacteria (Sharma *et al.*, 1997). If the thickness or composition of the mucus layer is altered, the effectiveness of the mucus layer as a barrier may be impaired increasing the susceptibility of gastrointestinal inflammation.

Yellowtail kingfish, *Seriola lalandi*, have been reported to suffer from sub-acute enteritis, termed ‗winter gut or red intestine syndrome‘, when plant proteins, such as soybean meal, were included in industry feeds when given at low water temperatures. However, there are few, if any, external symptoms; but with a higher mortality rate this winter gut syndrome

is of high concern to aquaculture companies as it has the possibility to limit the production of Yellowtail kingfish.

Overview of Aquaculture

Growth

The growth rate of the human population has shown no sign of slowing, nor has the demand for seafood. There will be an estimated 37 million tonnes of additional seafood required by the growing global population in the next 20 years (FRDC 2009). Since 1980, there has been relatively little change in the amount captured by the commercial fishery due to fully, or over exploited wild fish stocks. In contrast, since 1950, the global annual increase in production from aquaculture has been 8.8% (Fig. 1), far exceeding that of other livestock sectors which include: 4.9% for chicken, 3% for lamb, mutton and pig meats and 1% for beef and veal (Tacon, 2004). To supply the world‘s additional 37 million tonnes of seafood, an increased dependence on aquafeeds for aquaculture will be unavoidable.

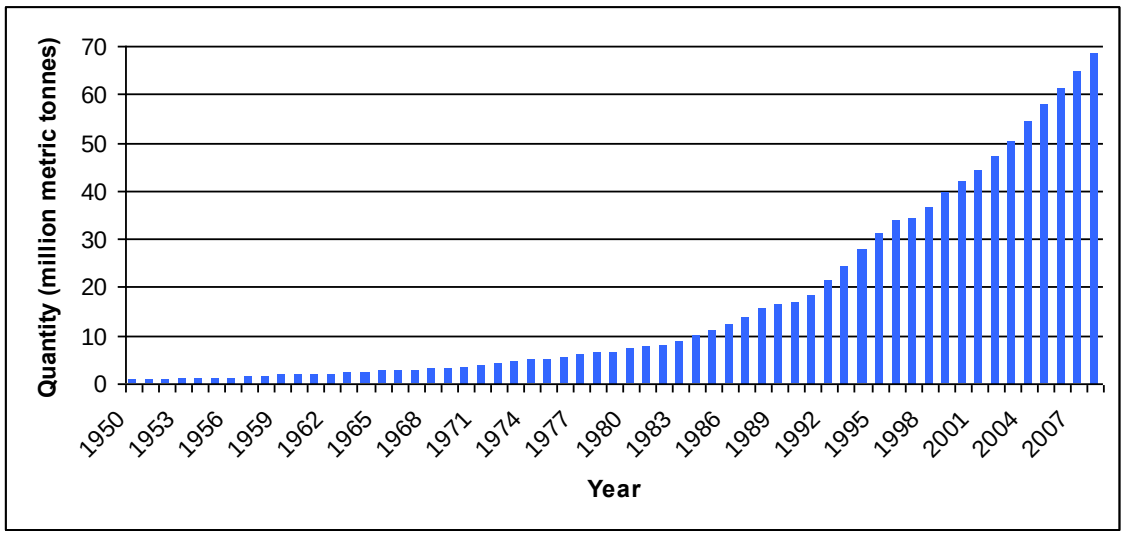

Figure 1 Reported global aquaculture production from 1950 to 2008, values from (FAO, 2010)

Sustainability

Fish meal and oil are derived from wild fish stocks; that are usually small, low trophic, pelagic fish which are unfavourable for human consumption as their flesh is unpalatable or has poor keeping abilities (Tacon and Metian, 2009). With the projected increased production of aquaculture and requirement of aquafeeds, the demand for fish meal and oil will soon exceed supply (Gatlin *et al.*, 2007). This will result in the price of fish meal increasing substantially and the forecasted growth in aquaculture production will become unsustainable. There has already been a substantial increase in the price of fish meal, with prices almost doubling from US$694 to US $1379 per tonne from 2005 to 2006 (Tacon and Metian, 2009), and increasing further to US $1705 per tonne in July 2010 (Fig. 2). Alternative protein sources will be required to meet the shortfall in supply of fishmeal if the expansion of aquaculture production is to continue.

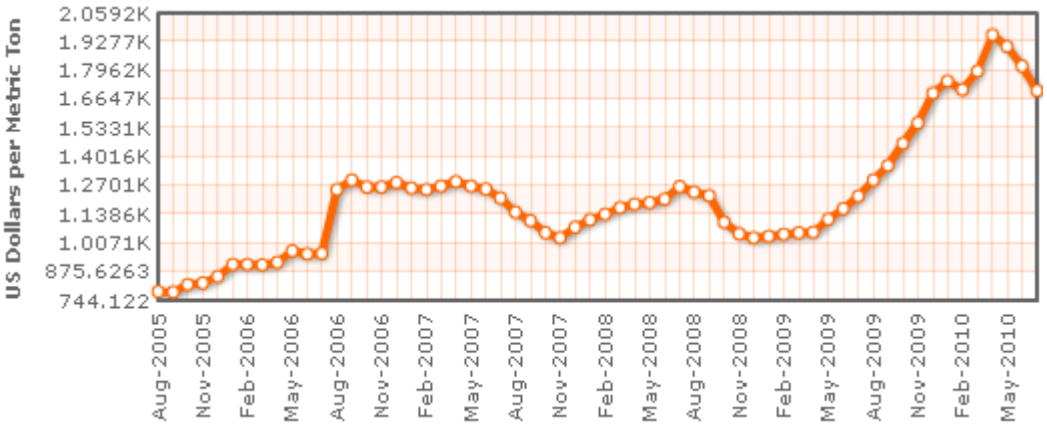

Figure 2 Price of fish meal August 2005 to May 2010 ($US/MT) (Mundi, 2010)

Yellowtail Kingfish in Aquaculture

Background

Yellowtail kingfish, *Seriola lalandi,* are marine, pelagic, schooling fish that inhabit temperate marine waters, often found associated with the coastline, reefs or offshore islands throughout the Pacific and Indian Oceans. They are a predatory species feeding at dusk and dawn on small fish, squid and crustaceans (PIRSA, 2002).Yellowtail kingfish are known to

grow to 70kg and reach a maximum length of 190cm, but are more commonly found at weights less than 15kg (PIRSA, 2002). They reach sexual maturity at approximately four to five years of age, spawning between October and January (PIRSA, 2002).

A number of species in the genus *Seriola,* including *Seriola dumerili* and *S. quinqueradiata,* are grown in aquaculture throughout Asia, South America and Australasia (Hilton *et al.*, 2008). Yellowtail kingfish have been grown in aquaculture in South Australia for over a decade (Fowler *et al.*, 2003); and is an important commercial species in many other countries (Hutson *et al.*, 2007, Fernandes and Tanner, 2008). In Japan the production of Yellowtail kingfish relies on the capture of wild juveniles, whereas in Australia the life-cycle of Yellowtail kingfish is closed, with juveniles produced from captive wild stock (Hilton *et al.*, 2008).

Suitability and Value in Aquaculture

Yellowtail kingfish have excellent flesh characteristics which has resulted in an increased demand in both the domestic and export market (Poortenaar *et al.*, 2001). Yellowtail kingfish are an ideal species for aquaculture due to their suitability for cage culture, exceptional feed conversion ratio and fast growth rate, being able to reach 3kg in 12 to 18 months (PIRSA, 2002).

The total production of Yellowtail kingfish in South Australia in 2005/06 was 1,500 tonnes increasing to approximately 3,370 tonnes in 2007/08 (Cleanseas, 2009). In contrast, the production of Southern Bluefin tuna in South Australia in 2005/06 was 8806 tonnes almost six times greater than Yellowtail kingfish, indicating the potential increase in future production of Yellowtail kingfish.

Issues

Temperature

Temperature is the most important environmental factor influencing growth rate and metabolism of fish (Quartararo *et al.*, 1998). Individual species have different optimal environmental conditions for biological processes.

In South Australia, Yellowtail kingfish are exposed to temperatures ranging from 10 to 24ºC. When Yellowtail kingfish were exposed to winter water temperatures, 12.6 ± 0.1°C, the time taken for feed to be voided from the digestive tract was reported to be between 36 to 48 hours (Miegel *et al.*, 2010). At higher water temperatures of 20.8 ± 0.4°C, similar to what Yellowtail kingfish are exposed to in summer, the digestive tract retention time was reduced to 12 to 16 hours (Miegel *et al.*, 2010). The reduced digestive tract retention time at lower water temperatures could have implication on the exposure to noxious substances in the diet of Yellowtail kingfish.

In a study conducted by (Quartararo *et al.*, 1998) the weight gain of snapper, *Pagrus auratus*, was investigated by providing two diets at two temperatures. Diet one contained 30% fish meal and 20% soybean meal which was compared to a second diet of 64% fish meal. The two diets were fed at ambient temperature, 13 ºC to 18.5ºC, and a temperature that on average was 4.3ºC higher, 18 ºC to 22.5ºC (Quartararo *et al.*, 1998). The weight gain of snapper fed diet one and two was 48.1±0.5g and 44.2±3.2g, respectively, at ambient water temperature. At the higher water temperature the weight gain of snapper fed diet one and two was 118.0±7.3g and 104.6±4.3g, respectively. For both diets there was no significant difference for weight gain, however, a significant difference was detected between the two water temperatures (Quartararo *et al.*, 1998). This suggests that water temperature has a greater effect on the growth rate of snapper compared to the protein source, demonstrating the increased efficiency of the digestive tract at higher temperatures.

Fish Meal

Feed is the largest cost for aquaculture companies. A carnivorous species may require 2.5 to 5 kilograms of feed to gain one kilogram (Naylor *et al.*, 2000). Fish meal is commonly included at 50% or higher in feed for carnivorous fish (Naylor *et al.*, 2000), and is an essential component of aquaculture feeds due to the high protein content, excellent amino acid profile, high digestibility and general lack of antinutrients (Gatlin *et al.*, 2007).

However, fish meal is a finite resource with the continued use in aquaculture feed not an ecologically sustainable or economic option. In 1988, aquaculture consumed 10% of the fish meal produced, which increased to 57% in 2006 (Tacon and Metian, 2009). The aquaculture industry‘s reliance on one ingredient places it at risk of the price, quality fluctuations and supply of fish meal.

Eight of the top twenty ocean species caught world wide between 1986 and 1997 were used in the production of fish meal. The eight species were Anchoveta, Chilean jack mackerel, Atlantic herring, Chub mackerel, Japanese anchovy, Round sardinella, Atlantic mackerel and European anchovy (FAO, 1999). If these species could be replaced by alternative plant proteins in aquaculture feeds it would not only be a more sustainable option but would significantly reduce costs (Quartararo *et al.*, 1998).

Soybean Meal and Soy Protein Concentrate

Background

Previously, legume seeds such as soybeans (van den Ingh *et al.*, 1991, Baeverfjord and Krogdahl, 1996), oilseed cake (Hossain and Jauncey, 1989), leaf meals (Wee and Wang, 1987) and numerous other alternative protein sources for fish meal have been included in experimental diets for fish. Soybean meal has been researched most extensively due to its high crude protein content, availability and relatively low cost when compared to the other plant protein alternatives (Francis *et al.*, 2001a).

Dietary inclusions of soybean meal are of limited success for carnivorous fish with species specific inclusion levels (Gatlin *et al.*, 2007). This is due to its lower protein content, lower carbohydrate digestibility and also due to the presence of a number of antinutritional factors when compared to fish meal (Tacon, 1995).

Antinutritive Properties

Antinutritional factors have detrimental effects on the growth rate and digestive system of fish. A number of different antinutritional factors are present in unprocessed soybean meal including oligosaccharides, saponins and trypsin inhibitors. Lectins are also present in soybean meal and have been found to interfere with nutrient absorption in the intestine of rats (Lajolo and Genovese, 2002). However, the effects of lectins on fish are yet to be identified but may be similar effect to observations in rats.

The concentration and type of oligosaccharides in soybean meal differs to fishmeal, of particular concern is the concentration of raffinose and stachyrose. Raffinose and stachyrose are indigestible to fish and have been linked to a reduction in growth rate (Refstie *et al.*, 1998) and the development of sub-acute enteritis in salmonoid species (van den Ingh *et al.*, 1991, van den Ingh *et al.*, 1996).

Saponins are glycosides of steroids. When isolates were included in fish diets they were found to increase the permeability of intestinal mucosal cells, facilitating the uptake of substances that are normally not absorbed while inhibiting active transport of substances that are normally absorbed (Johnson *et al.*, 1986).

A number of experiments have been conducted on the inclusions of saponins in the diets of fish with varying effects on the histology and growth rate. Rainbow trout and Chinook salmon that were given 1.5g of saponins per kilogram had similar damage to the intestinal mucosa when compared to fish that were given raw soybean meal (Bureau *et al.,* 1998). However, with a dietary inclusion of 400mg of saponins per kilogram, the level of

saponins found in soybean meal, no detrimental effects on the histology of Atlantic salmon hind gut were observed (Krogdahl *et al.*, 1995). In contrast, a significant increase in the growth rate of common carp occurred when saponins were continuously available compared to a control diet (Francis *et al.*, 2001b).

Trypsin inhibitors are relatively stable to heat and acid treatment, they act by blocking the activity of either trypsin or a chymotrypsin (Norton, 1991). When soybean meal undergoes heat treatment, the concentration of trypsin inhibitors is reduced below 5mg/g. At this level most fish are able to compensate by increasing their trypsin synthesis (Francis *et al.*, 2001a).

Processing

Processing reduces the levels of antinutritional factors, increasing the nutritional profile of soybean products (Table 1). Soy protein concentrate is the product of soybean meal after processing by alcohol washing. Soybean meal has a lower concentration of ten essential amino acid compared to that of fish meal, while the concentration of amino acids in soy protein concentrate may exceed that of fish meal (Gatlin *et al.*, 2007).

Table 1 Antinutrional factors present in soy products with increasing processing compared to fishmeal (Peisker, 2001)

Antinutrtional Factors	Soybean Seed	Soybean Meal	Soy Protein Concentrate	Fish Meal
Oligosaccharides (%)	14	15	<4	-
b-conglycinin (mg/g)	75	25	<0.1	-
Glycinin (mg/g)	175	55	<0.1	-
Lectins (mg/kg)	125	125	<0.1	-
Phytic acid-P (%)	0.6	0.6	0.6	-
Raffinose (%)	0.9	1.1	<0.2	-
Saponins (%)	0.5	0.6	0	-
Stachyose (%)	4.2	5	2	-
Trypsin inhibitor activity (mg/g CP)	53	6	2.5	-

The growth rate of Rainbow trout when fish meal was replaced with 50% soy protein concentrate was similar to Rainbow trout fed 100% fish meal (Mambrini *et al.*, 1999). The alcohol soluble compounds present in soybean meal, which have a reduced concentration in soy protein concentrate, are thought to be the cause of sub-acute enteritis (Refstie *et al.*, 2005). However, due to the high processing and production costs associated with soy protein concentrate, it is not yet an economically viable option for large scale use in aquafeeds (Gatlin *et al.*, 2007).

Sub-Acute Enteritis

The inclusion of soybean meal has occurred for a number of species including Channel catfish (Evans *et al.*, 2005), Chinook salmon (Fowler, 1980), Atlantic salmon (Baeverfjord and Krogdahl, 1996) and Rainbow trout (Merrifield *et al.*, 2009). Dietary inclusions of soybean meal are species specific, an inclusion of less than 10% soybean meal caused sub-acute enteritis in the hindgut of Atlantic salmon, while an inclusion of 57% soybean meal in the diet of hybrid tilapia was given before this condition was induced (Stone, 2007).

In a study investigating the effect of soybean meal on gastrointestinal morphology, Rainbow trout were fed two diets for 16 weeks, a standard fish meal diet and a dietary inclusion of 50% soybean meal. The 50% soybean meal treatment showed histological and functional changes in the gastrointestinal tract. The distal intestine showed an increased proportion of inflammatory cells and microvilli that were significantly shorter and less dense when compared to the control group (Merrifield *et al.*, 2009). These effects were also apparent for Atlantic salmon in conjunction with a loss of normal supranuclear vacuolization in the epithelial cells, a widening of the lamina propia and a shortening of the primary and secondary mucosal folds (Fig. 3) (Baeverfjord and Krogdahl, 1996).

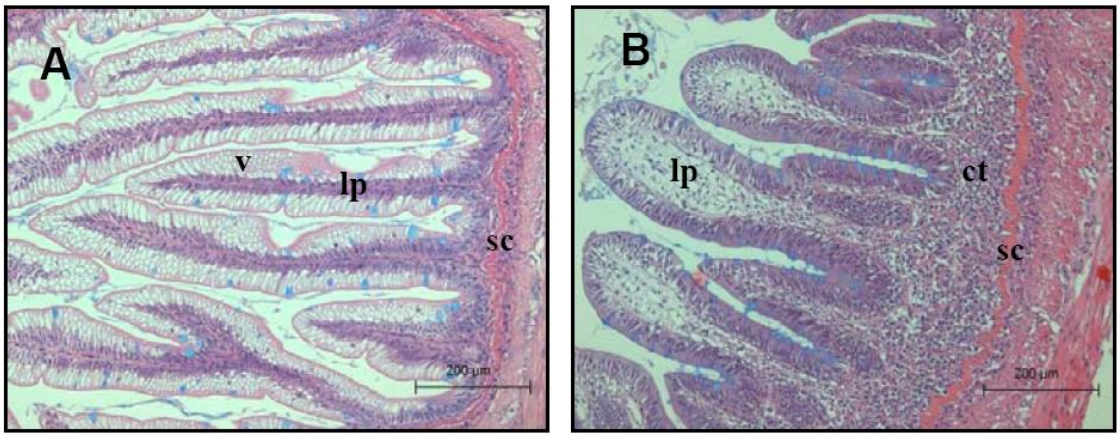

Figure 3 A) Normal morphology in the hindgut of an Atlantic salmon. B) Dietary inclusion of soybean meal inducing sub-acute enteritis in the hindgut of Atlantic salmon (vacuoles: v, lamina propria: lp, connective tissue: ct, and stratum compactum: sc). (Uran, 2008).

In contrast to carnivorous species such as Rainbow trout and Atlantic salmon, when a dietary inclusion of 45% soybean meal was given to Channel catfish no alterations, outside of the normal limit, to the histology of the hindgut were observed (Evans *et al.*, 2005). The effect seen on the histology of the hindgut with inclusions of soybean meal varies between species of fish, and appears to be dependent on the physiological adaptations of the specific species.

Due to the inflammation in the gastrointestinal tract, energy that would be used for growth is instead used to repair damaged tissue. This has implications for the aquaculture industry as there is a reduction in feed conversion efficiency resulting in an increased cost of production (Sweetman *et al.*). Sub-acute enteritis may also lead to individuals being more susceptible to bacterial infections and secondary diseases due to an increased permeability of the epithelium (Urán, 2008).

Mucus Layer

Goblet Cells

Goblet cells are highly polarized exocrine cells created at the base of crypts of the gastrointestinal epithelium (Specian and Oliver, 1991). The primary function of goblet cells is to synthesise and secret mucin, a major component of the mucus layer (Sharma *et al.*, 1997).

Mucin is secreted by goblet cells by two different processes, baseline and accelerated secretion (Deplancke and Gaskins, 2001). Baseline secretion is achieved through exocytosis of a single mucin granule resulting in a slow and continuous secretion of mucin (Specian and Oliver, 1991). In contrast, accelerated secretion occurs in response to a number of different stimuli resulting in a rapid discharge of all stored mucin in goblet cells. Likely stimuli for accelerated secretion include cholinergic stimulation, intestinal anaphylaxis, and chemical and physical irritation (Specian and Oliver, 1991).

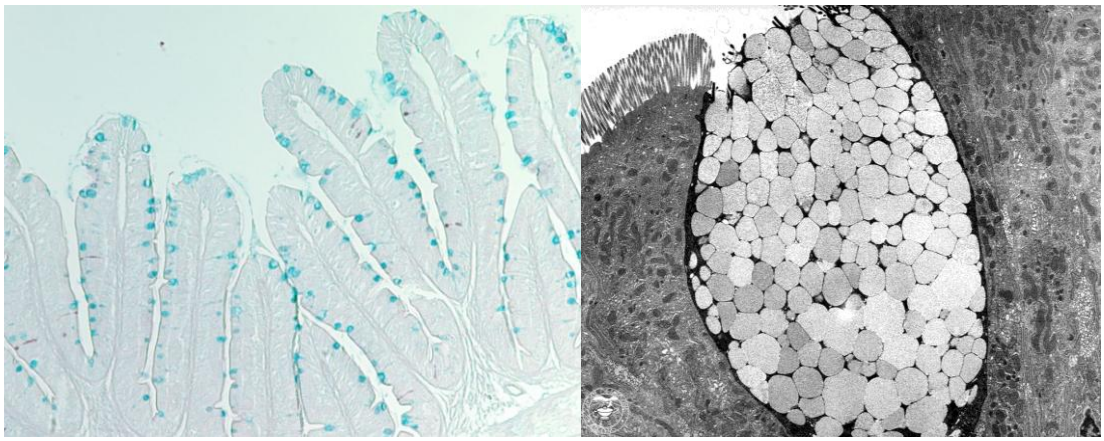

Figure 4 A) Goblet cell distribution in the villi of freshwater Nile Talapia (*Oreochromis niloticus*), B) electron micrograph of mucin granules in a goblet cell (right) (Pictures courtesy of Rebecca Forder)

Mucins

Mucins are high molecular weight glycoproteins comprising a protein core with branching oligosaccharide chains attached by glycoside bonds (Lievin-Le Moal and Servin, 2006). The protein core of the mucin molecule has a variable number of tandem repeats, made

up of a high proportion of serine, proline and threonine, these act as sites for attachment of oligosaccharides via the sugar N-acetyl-galactosamine. The oligosaccharide chains are attached to the protein core in a "bottle brush" fashion (Allen and Pearson, 1993). This region of the mucin molecule is highly heterogenous and complex; with either linear or branched and neutral or acidic carbohydrate subunits (Allen and Pearson, 1993).

Mucin molecules are co-packaged in goblet cells with multivalent cations, believed to be calcium ions, which act to shield the negative charge of the mucin molecule. When a mucin molecule is exposed to the luminal environment, the cation diffuses into the luminal fluid and the mucin molecule is rapidly hydrated. This results in a rapid expansion, up to 500 times the original volume in under 50 milliseconds (Verdugo, 1990). The expansion of mucin is due to the hydrophilic nature of the oligosaccharide chains and the loss of the cation resulting in an increase in the hydrostatic repulsion between mucin molecules (Strous and Dekker, 1992).

Mucus Layer

Composition

The mucus layer is a viscous gel that covers the majority of the mucosal surfaces of the gastrointestinal, respiratory and genitor-urinary tract (Turck *et al.*, 1993). In the intestine the composition of the mucus layer is a balance between degradation by microflora, that use mucin as an energy and nutrient source, and processes of digestion with the secretion by goblet cells (Sharma *et al.*, 1997, Shirazi *et al.*, 2000). The mucus layer is a mixture of water, cellular macromolecules, electrolytes, micro-organisms, enzymes, sloughed cells and mucins (Neutra *et al.*, 1987).

Function

The mucus layer is the first line of defence for the gastrointestinal epithelium (Sharma *et al.*, 1997). It is a major barrier for the intestine forming a surface network layer above the

villi, separating epithelial cells from the luminal environment (Claustre *et al.*, 2002, Smirnov *et al.*, 2004). The mucus layer protects the epithelium from mechanical damage, adhesion of bacteria, toxic substances, destructive enzymes and corrosive chemicals (Gupta, 1989, Specian and Oliver, 1991).

Prevention of mechanical damage to the epithelium is due to the excellent lubricative property of the mucus layer. When mucus is rapidly sheared, the secondary bonds are broken between layers of mucus, forming a slippage plane (Cone, 1999). The slippage plane is formed due to the loosely adherent mucus layer sliding past the mucus layer that is firmly attached to the epithelium which results in no adverse effects to the epithelium (Corazziari, 2009).

The mucins protein core is comprised of a heavily glycosylated region, which is hydrophilic, and a sparsely glycosylated region, which is hydrophobic (Allen and Pearson, 1993). Due to both hydrophilic and hydrophobic regions, there are few substances, with which a mucin molecule cannot form low affinity bonds. Substances that are rapidly bound by mucin molecules include destructive enzymes, extracellular matrix and pathogenic organisms (Strous and Dekker, 1992, Cone, 2009). Unless the production of mucin is inhibited, the mucus layer provides a renewable surface that can attach to and facilitate the removal of detrimental foreign particles (Shephard, 1994).

Interactions

Alteration to Mucus and Intestinal Inflammation

A number of gastrointestinal diseases are associated with a degraded mucus layer or alteration to the biochemical characteristics of mucins in the mucus layer (Sharma *et al.*, 1995, Rhodes, 1989). A reduction in the protective ability of the mucus layer may result in an increased vulnerability to ulcerative colitis and Crohn‘s disease in humans (Strous and

Dekker, 1992, Pullan *et al.*, 1994), necrotic enteritis in chickens (Collier *et al.,* 2008), and detrimental compounds, such as saponins, in the gastrointestinal tract of fish (Bureau *et al.*, 1998).

Ulcerative colitis, an inflammation in the human colon, is thought to be as a result of a reduction in the thickness of the mucus layer, which is associated with a reduced number of goblet cells (Pullan *et al.*, 1994). However, inflammation in the gastrointestinal tract is not always due to a reduction in the thickness of the mucus layer.

Crohn‘s disease has a relatively thicker mucus layer with no significant change to the goblet cell numbers (Pullan *et al.*, 1994, Rhodes, 1997). In this case, the organism is still susceptible to inflammation; however, no reduction in the thickness of the mucus layer occurs, indicating the protective ability of the mucus layer may be compromised due to an alteration in the composition of mucin. The composition may be compromised through a reduction in the heterogeneity of the oligosaccharide region in the mucin molecules resulting in a reduction in the available bonding sites in the mucus layer (McGuckin *et al.*, 2009, Shirazi *et al.*, 2000).

Necrotic enteritis in poultry is caused by the over proliferation of the bacteria, *Clostridium perfringens*. However, *C. perfringens* is not detrimental until the mucosa is compromised by predisposing factors such as *Eimeria* parasites. Inflammation caused by *Eimeria* parasites is thought to allow *C. perfringens* to rapidly replicate or to produce toxins (Van Immerseel *et al.*, 2009). At present, the role that the mucus layer and mucins play in the infection of *C. perfringens* is unknown, with studies suggesting that the increase in mucin secretion from initial exposure to *Eimeria*, may allow *C. perfringens* to utilize mucin as a nutritional substrate, providing a growth advantage to the species (Collier *et al.,* 2008).

Alterations to the composition or thickness of the mucus layer have resulted in inflammation in the gastrointestinal region in a number of species. The degradation of the

mucus layer and the role it plays in the protection against sub-acute enteritis in fish has not been studied. However, because the pathology of sub-acute enteritis is similar to conditions of intestinal inflammation in other species, it is speculated that alterations to the composition or thickness of the mucus layer may be involved in its pathogenesis.

Mucus and Diet

The thickness of the mucus layer varies between regions in the gastrointestinal tract; however, the thickness of the mucus layer within each region is relatively similar, indicating optimal mucus thickness for protection and function (Corfield *et al.*, 2001). Both synthesis and degradation of the mucus layer are natural processes; however, a balance between these processes is required to protect the epithelium against potential harmful organisms and compounds present in the diet (Corfield *et al.*, 2001).

The gastrointestinal content is primarily determined by the non-absorbed nutrients from the diet. This influences the survival of pathogens, the composition of the autochthonous, native, microflora and the functioning of the mucus layer (Bovee-Oudenhoven and van der Meer, 2001). Mechanical damage (Szentkuti and Lorenz, 1995) and alteration to the mucin composition (Sharma *et al.*, 1997) are also influenced by diet.

A study investigating the effect of a dietary inclusion of soybean meal on Rainbow trout, found an alteration to both the autochthonous, native, and allochthonous, foreign, micro-biota in the gut (Merrifield *et al.*, 2009). Alterations to the bacterial colonies in the gastrointestinal tract were also evident in rats following inclusions of proteins from casein, whey and soy in the diets, which was hypothesised to be an indirect cause for the reduced mucus layer thickness (Toden *et al.*, 2007).

When rats were subjected to high fibre diets there was a reduction in the mucus layer thickness and an alteration to the mucin composition (Szentkuti and Lorenz, 1995). The reduction in mucus layer thickness was due to an increase in mechanical damage from the

movement of chyme when fed a high fibrous diet, which was thought to wipe away the outer section of the mucus layer (Szentkuti and Lorenz, 1995). A similar study that assessed that effect of fibre in the diet of rats concluded that there was an increased secretion of acidic mucins; which has implications on the carbohydrate and amino acid composition in the mucus layer (Enss *et al.*, 1994).

During times of malnutrition, the maintenance of mucus synthesis has a lower priority than preservation of the digestive surface (Neutra *et al.*, 1974). A reduction in mucus layer thickness occurred when rats were subjected to nutritional deprivation (Sherman *et al.*, 1985). A thinner mucus layer was also observed in chickens where they were starved with a resultant decrease in body weight (Smirnov *et al.*, 2004).

A number of studies have been observed sub-acute enteritis in fish when fed dietary inclusions of soybean meal (Evans *et al.,* 2005, Fowler, 1980, Baeverfjord and Krogdahl, 1996, Merrifield *et al.,* 2009). However, the initiating causes of sub-acute enteritis or the effect of dietary soy products on mucus layer dynamics remains unknown.

Summary

Aquaculture companies are being pushed towards a more economical and ecological sustainable protein source due to a substantial increase in the price and a reduction in the availability of fish meal. Currently, soybean meal is under scrutiny as an alternate protein source due to its low cost, relatively high protein content and availability (Francis *et al.*, 2001a). However, dietary inclusions of soybean meal have been linked to sub-acute enteritis in the hindgut of a number of different fish species (Evans *et al.*, 2005, Fowler, 1980, Baeverfjord and Krogdahl, 1996, van den Ingh *et al.*, 1991). Inclusions of soybean meal in feeds for Yellowtail kingfish are becoming common, with high dietary inclusion levels of soybean meal coupled with low water temperatures possibly contributing to sub-acute enteritis, termed ‗winter gut or red intestine syndrome', in the hind gut of Yellowtail kingfish.

The pathogenesis of sub-acute enteritis in Yellowtail kingfish is hypothesised to be dependent on water temperature and the dietary inclusion level of soybean meal; however, this is still yet to be determined experimentally. The role that the mucus layer plays in the protection against sub-acute enteritis is unknown for fish. However, based on experimental data of gastrointestinal inflammation in other species we hypothesise that sub-acute enteritis in Yellowtail kingfish is caused by a decrease in the efficacy of the mucus layer in the hindgut through alterations to the thickness or composition.

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PART B: THESIS

Abstract

Yellowtail kingfish (*Seriola lalandi,* Carangidae) have been farmed for over a decade in sea cages in the waters of Spencer Gulf, South Australia. Substantial fluctuations of the water temperature in Spencer Gulf occur, reaching 24ºC in summer and dropping below 12ºC in winter. Inclusions of soybean meal in feeds for Yellowtail kingfish are becoming common. High dietary inclusions of soybean meal coupled with low water temperatures are thought to contribute to the development of sub-acute enteritis in the hindgut of Yellowtail kingfish. Prior to this study, the role the mucus layer plays in protecting the underlying mucosa had not been investigated in fish.

In this study, fish were fed increasing dietary inclusion levels of solvent extracted soybean meal (SE SBM), to apparent satiation twice daily, at water temperatures of 18ºC and 22ºC for 34 days. At the conclusion of the study, the intestinal tract was removed, with no fish exhibiting visual features of hindgut inflammation. Samples were collected for histological evaluation, revealing a significant reduction in mucus layer thickness in the hindgut of fish fed increasing dietary inclusion levels of SE SBM. Water temperature had a significant effect on mucin composition. A more profound increase in neutral mucins in the hindgut was observed at 18ºC, while a more profound increase of acidic mucins was evident at 22ºC. Fish fed 20% and 30% dietary inclusions of SE SBM at 18ºC had a significant increase in goblet cell number. Although sub-acute enteritis was not induced in this study, it is evident that the intestinal barrier was compromised. Based on observations from this study, SE SBM inclusion levels in the diet for Yellowtail kingfish of this size range should be restricted to 10% at 18°C and 20% at 22°C. Further studies are required to assess alteration to intestinal morphology and the development of sub-acute enteritis in Yellowtail kingfish reared in colder waters indicative of those experienced during winter months.

Keywords: mucin composition, mucus layer, sub-acute enteritis, yellowtail kingfish

Introduction

Yellowtail kingfish are a promising aquaculture species, with an excellent feed conversion ratio, growth rate and flesh characteristics (PIRSA, 2002). Recently, an increased demand for Yellowtail kingfish in both the domestic and export market has occurred (Poortenaar *et al.*, 2001), resulting in an increase in production in South Australia, from 1,500 tonnes in 2005/06 to 3,370 tonnes in 2007/08 (Cleanseas, 2009).

Fish meal is the primary component of Yellowtail kingfish feed due to its high protein content, digestibility and palatability and lack of antinutrients, such as oligosaccharides and saponins (Gatlin *et al.*, 2007). However, fish meal is a finite resource with a static annual production of approximately seven million metric tonnes (Hardy *et al.*, 2002). Recently there has been a substantial increase in the price of fish meal, with prices rising from US $694 per tonne in 2005 to US $1705 in 2010 (Mundi, 2010). The price of fish meal is expected to increase further due to an increased demand. As a result, aquaculture companies are being pushed towards using more economic and ecologically sustainable protein sources.

A number of alternative protein sources to replace fish meal have been included in experimental diets for fish including oilseed cake (Hossain and Jauncey, 1989), leaf meals (Wee and Wang, 1987) and legume seeds (van den Ingh *et al.*, 1991, Baeverfjord and Krogdahl, 1996). Currently, soybean meal is of interest as an alternative protein due to its high crude protein content, relatively low cost and availability (Francis *et al.*, 2001a). However, dietary inclusions of soybean meal for aquaculture species are often limited by high concentrations of antinutritional factors including oligosaccharides, β-conglycinin, glycinin, lectins, phytic acid-P, saponins and trypsin inhibitors (Peisker, 2001 Francis *et al.*, 2001, Gatlin *et al.,* 2007).

Dietary inclusion of soybean meal has been found to induce a condition first described in Atlantic salmon (*Salmo salar*, Salmonidae) as sub-acute enteritis of the distal epithelial

mucosa (Baeverfjord and Krogdahl, 1996). This condition is characterised by an increased proportion of inflammatory cells, decreased height and density of villi and microvilli (Merrifield *et al.*, 2009), a loss of normal supranuclear vacuolization in the epithelial cells, a widening of the lamina propria and a shortening of the primary and secondary mucosal folds (Fig. 1) (Baeverfjord and Krogdahl, 1996).

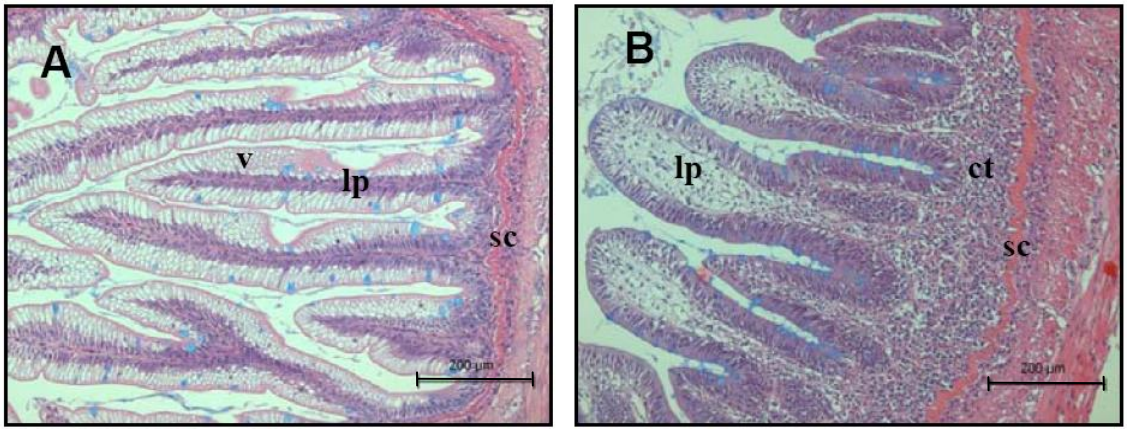

Figure 1 A) Normal morphology in the hindgut of an Atlantic salmon. B) Soybean meal induced sub-acute enteritis in the hindgut of Atlantic salmon (vacuoles: v, lamina propria: lp, connective tissue: ct, and stratum compactum: sc) (Uran, 2008).

The inclusion level of soybean meal in the diet is species specific. The dietary inclusion of 10% genetically modified (GM) or 10% non-GM full fat soybean meal has been found to induce sub-acute enteritis in the hindgut of Atlantic salmon (Sanden *et al.*, 2005). In contrast, there were no observed alterations to the hindgut morphology, and no evidence of sub-acute enteritis when Channel catfish (*Ictalurus punctatus*, Ictaluridae) were fed diets containing 45% raw or heat treated full fat soybean meal (Evans *et al.*, 2005).

The mucus layer is the first line of defence for the gastrointestinal epithelium (Sharma *et al.*, 1997), separating epithelial cells from the luminal environment (Claustre *et al.*, 2002, Smirnov *et al.*, 2004), providing protection from pathogens, destructive enzymes and corrosive chemicals (Gupta, 1989, Specian and Oliver, 1991). The mucus layer is a mixture of

water, cellular macromolecules, electrolytes, bacteria, enzymes, sloughed cells and mucins (Neutra *et al.*, 1987). Mucins, a major component of the mucus layer, are synthesised and secreted by goblet cells (Sharma *et al.*, 1997). They are high molecular weight glycoproteins comprising a protein core with branching oligosaccharide chains attached by glycoside bonds (Lievin-Le Moal and Servin, 2006). The oligosaccharide chains are highly heterogonous and complex; with either linear or branched, neutral or acidic carbohydrate subunits (Allen and Pearson, 1993). Their highly heterogenous nature provides a vast array of potential binding sites for intestinal bacteria, thereby retarding access of micro-organisms to the mucosal surface reducing colonisation and favouring their removal.

Alterations to the thickness or composition of the mucus layer impair its effectiveness as a barrier, increasing the susceptibility of the gastrointestinal tract to inflammation. The role the mucus layer plays in protecting the underlying mucosa has been identified in a number of gastrointestinal inflammatory disorders including ulcerative colitis in humans (Pullan *et al.*, 1994), Crohn‘s disease in rodent models of human disease (McGuckin *et al.*, 2009, Shirazi *et al.*, 2000) and necrotic enteritis in poultry (Collier *et al.*, 2008, Van Immerseel *et al.*, 2009).

At present, there is no information on mucus layer structure, composition or mucosal protection by the mucus layer and subsequent pathogenesis of sub-acute enteritis in Yellowtail kingfish. Thus, the primary aim of this study was to investigate and evaluate the efficacy of the intestinal mucus layer through changes in mucin synthesis and secretion associated with increasing dietary inclusion levels of SE SBM at optimal (22°C) and sub-optimal (18°C) water temperatures in Yellowtail kingfish.

Materials and Methods

Experimental work was conducted in the Nutrition Laboratory at the South Australian Research and Development Institute (SARDI) Aquatic Science Centre, West Beach, South Australia. Samples for this study were collected from two interrelated Australian Seafood CRC projects (Sustainable Feeds and Feed management for Yellowtail kingfish project (2009/728); Nutritional factors influencing the performance of Yellowtail kingfish cultured at low temperatures (JQ001)). Prior to being used in this study, fish were housed in 5,000L tanks at SARDI Aquatic Science Centre, West Beach**.** Experimental work was conducted in November and December 2010. A total of 384 juvenile Yellowtail kingfish were used in this study and were sourced from the Arno Bay hatchery, Cleanseas Tuna Ltd, SA.

Animal Housing and Feeding

Upon commencement of the study, fish weighing between 20 and 25 grams were selected from a larger population and transferred by net into 80L of seawater containing 20 mg L^{-1} AQUI-S (AQUI-S New Zealand Ltd., Lower Hutt, New Zealand) and allocated by systematic interspersion to one of sixteen, 700L tanks. Twenty four Yellowtail kingfish were stocked in each tank; with a similar initial biomass for all tanks.

Eight treatments were investigated in this study; four diets, a control diet (46% fish meal and 0% SE SBM) and three diets with fish meal replaced with 10%, 20%, 30% inclusions of SE SBM. All feeds were prepared using a 3mm die with steam cooking extrusion at the SARDI Australasian Experimental Stockfeed Extruded Centre (AESEC) at Roseworthy, South Australia. Each diet was fed at sub optimal (18ºC) and optimal (22ºC) water temperatures, with two replicate tanks for each treatment.

Fish were supplied with recirculating seawater and had a fixed photoperiod of 14 h light: 10 h dark. Throughout the study, fish were fed to apparent satiation twice daily at 0900

and 1530 for 34 days. The water temperature, dissolved oxygen, pH and ammonia concentration was monitored daily and salinity monitored weekly.

Sample Collection

At the conclusion of the trial, fish were fasted for 24 hours before being anaesthetised in 20mgL^{-1} AQUI-S (AQUI-S New Zealand Ltd., Lower Hutt, New Zealand) in 80L of seawater and dispatched with a spike to the brain. Fish were then dissected and the gastrointestinal tract removed. The distal half of the hindgut (Fig. 2) was collected from three fish per tank (n = 6 fish/treatment) and cut into three segments for three fixation methods.

The distal half of the hindgut was opened longitudinally onto biopsy paper and cut in half, one half was fixed in 10% buffered formalin, the other half was fixed in Carnoy‘s solution (see Appendix 1). Samples fixed in Carnoy‘s solution were transferred to 100% ethanol after 2 hours and transported from SARDI Aquatic Science Centre to the University of Adelaide, Roseworthy Campus and immediately processed before being embedded in paraffin wax (Matsuo *et al.*, 1997). Buffered formalin samples were fixed for 24 hours, processed and embedded in paraffin wax.

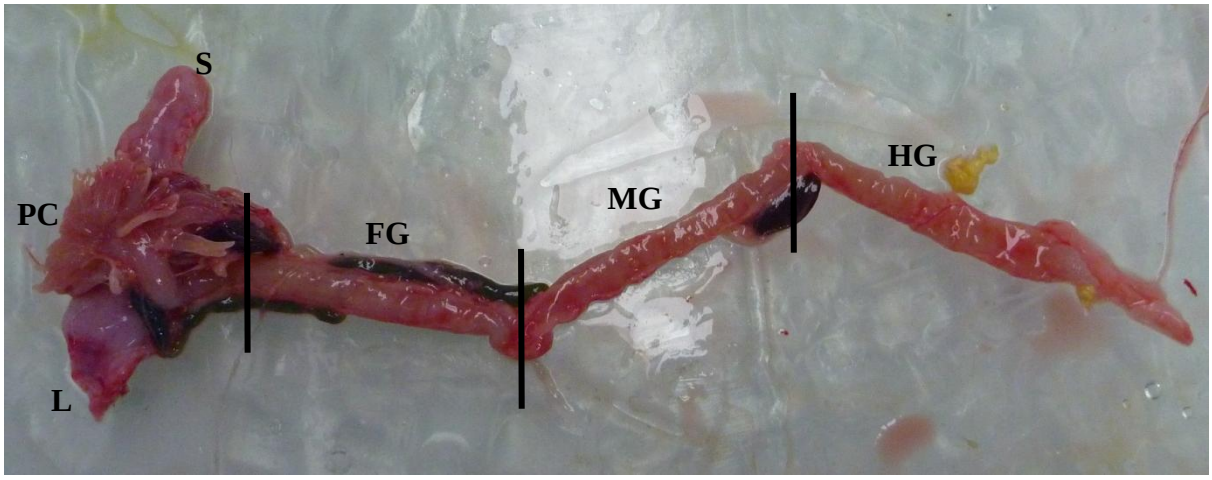

Figure 2 Gastrointestinal tract of a Yellowtail kingfish, *Seriola lalandi*, (stomach: S, pyloric caeca: PC, liver: L, foregut: FG, midgut: MG, hindgut: HG)

Histology

All tissue samples were cut at 5µm on a Thermo Scientific Microm HM340E microtome (Microm International GmbH, Waldorf, Hessen, Germany), floated onto Starfrost® glass slides and allowed to stand for greater than 24 hours before being stained.

Intestinal Morphology

Mucosal architecture was more pronounced in tissue fixed in buffered formalin. Slides were stained with PAS/AB pH 2.5, photomicrographs were taken at 40X magnification using an Olympus WH B10X\20 microscope (Olympus, Tokyo, Japan) and Colorview Soft imaging System CX41 camera (2048 x 1538 pixel resolution) (Soft Imaging System, Brook-Anco Corp, Rochester, NY)**.** Villus height, villus width and lamina propria area were measured for approximately 20 villi per section depending on the quality of the section using the image analysis program, Video Pro® (version 6.2.1.0, Leading Edge Pty. Ltd., Australia). The villus height (VH; µm) was measured from the tip of the villus to the base of the villus. The apical (AW; µm) and basal (BW; µm) width of the villus was measured in order to calculate approximate villus area (VA; μm^2). Villus area was calculated using the formula VA= ((AW+BW)/2)x(VH). The lamina propria area was measured from the base of the villus to the tip of the lamina propria and expressed as a percentage of total villus area.

Mucus Layer

Sections were stained with periodic acid-schiffs/alcian blue pH 2.5 (PAS/AB pH 2.5; see Appendix 2) and mounted in DPX mounting medium (Ajax Finechem Pty Ltd, Taron Point, NSW, Australia). Five photomicrographs were taken at randomly selected regions throughout the sample at 100X magnification using an Olympus WH B10X\20 microscope (Olympus, Tokyo, Japan) and Colorview Soft imaging System CX41 camera (2048 x 1538 pixel resolution) (Soft Imaging System, Brook-Anco Corp, Rochester, NY)**.** Five random measurements of mucus layer thickness were taken between villi for each photo using the

image analysis program, Video Pro® (version 6.2.1.0 , Leading Edge Pty. Ltd., Australia), resulting in 25 measurements for mucus layer thickness for each fish a using method similar to those described by Matsuo *et al.* (1997).

Goblet Cell Number and Mucin Composition

Goblet cell number and mucin composition were measured in individual villus using buffered formalin fixed sections as described previously. Two staining techniques were used to differentiate between neutral and acidic mucins as well as sialylated and sulphated acid mucin sub-types. PAS/AB pH 2.5 was used to detect neutral mucins (pink) and acidic mucins (blue). High iron diamine/alcain blue pH 2.5 (HID/AB pH 2.5; see Appendix 2) was employed to detect sialylated mucins (blue) and sulphated mucins (dark brown). Image J® was used to calculate the number of goblet cells per millimetre villus height (mm). For PAS/AB pH 2.5 and HID/AB pH 2.5 individual counts were obtained for goblet cells stained either blue or pink and blue or brown, respectively. Goblet cells stained both pink and blue or brown and blue were counted separately and termed ~~"i~~intermediate". The summed values of neutral and intermediate goblet cells in slides stained with PAS/AB pH2.5 provided a total count of neutral goblet cells, the summed values of all cells counted in slides stained with HID/AB pH2.5 provided a total count of acidic goblet cells.

Statistics

The homogeneity of variance and normality of data was assessed from the standardized residuals against the predicted mean plot in order for a general linear model to be fitted. Final fish weight was included as a covariate, but was removed in the final model for all variables measured as it was determined to be non-significant ($P>0.05$). Total goblet cell numbers were included as a covariate for goblet cells containing neutral and acidic mucins. A significance level of $P<0.05$ was used. All data are expressed as means ± standard error of the mean.

Results

Growth

Figure 3 shows the greater effect of water temperature, than dietary inclusion of SE SBM, on the final weight gain of Yellowtail kingfish. Fish at 22ºC had significantly higher weight gain compared to fish at 18ºC for all dietary treatments.

Fish fed 0%, 10% and 20% SE SBM at 18ºC, displayed similar weight gains. However, significantly lower weight gains were observed in fish fed a 30% dietary inclusion of SE SBM compared to controls and fish fed a lower dietary inclusion of SE SBM. Similar results were observed in fish reared at 22ºC. No significant differences in weight gain were observed for fish fed 0%, 10% and 20% SE SBM at 22ºC. In addition, no significant difference in weight gain were observed for fish fed 20% and 30% SE SBM, however, a significant reduction in weight gain was observed between fish fed the 30% dietary inclusion level of SE SBM and fish fed 0% and 10% SE SBM.

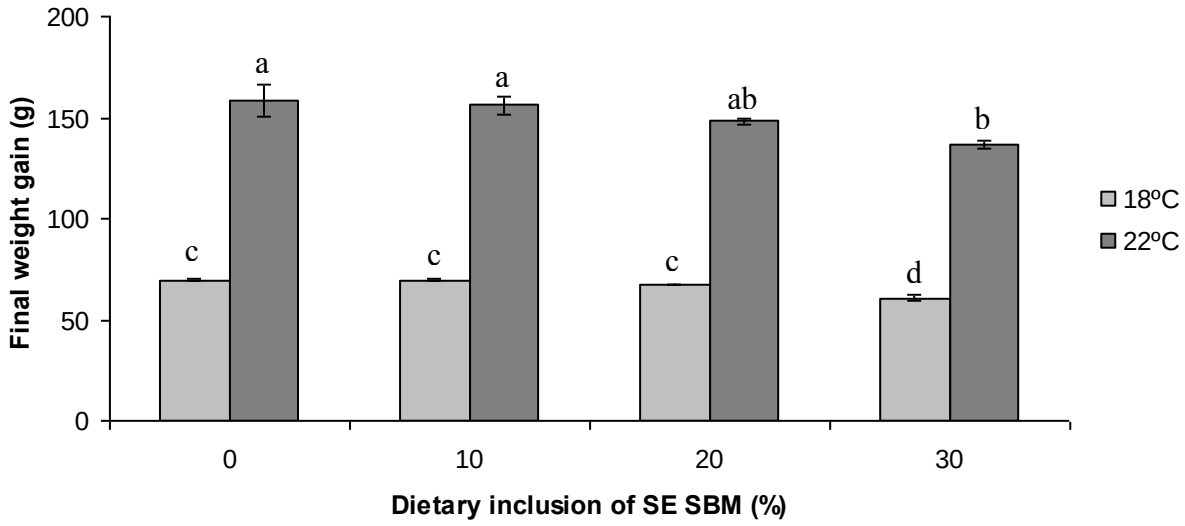

Figure 3 Final weight gain of Yellowtail kingfish given increasing dietary inclusion levels of SE SBM at 18ºC and 22ºC. Means with different superscripts were significantly different (n= 2 replicate tanks).

Villus Height and Area

Villi in the hindgut of Yellowtail kingfish were long, slender and branched (Fig. 4). Villi appeared similar in all treatments. Water temperature and dietary inclusion level of SE SBM had no significant effect on villus height or villus area.

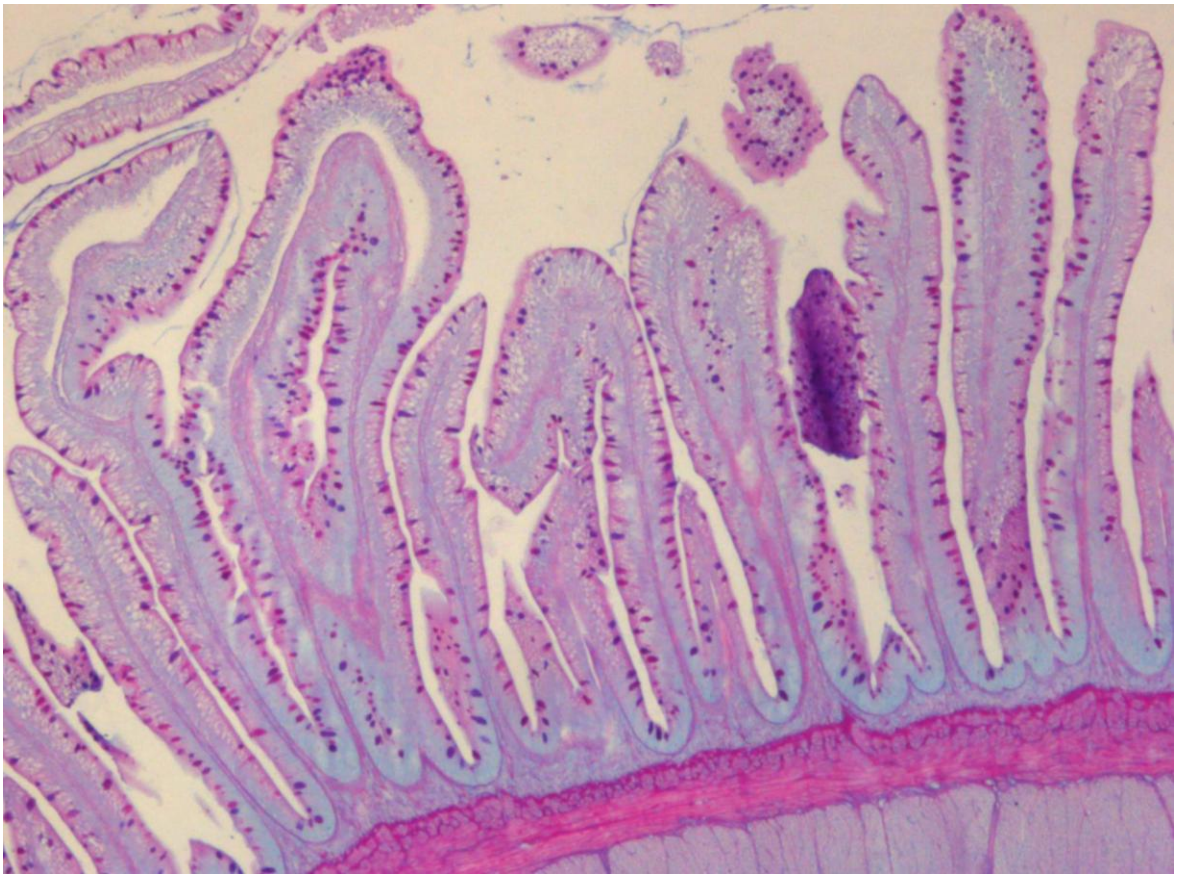

Figure 4 Hindgut morphology of Yellowtail kingfish fed 0% SE SBM at 22ºC (40X).

Lamina Propria Area

Dietary inclusion level of SE SBM had no significant effect on the lamina propria proportion of the villus area. A significantly greater lamina propria proportion of villus area was observed in fish at 22ºC compared to fish at 18ºC (Fig. 5).

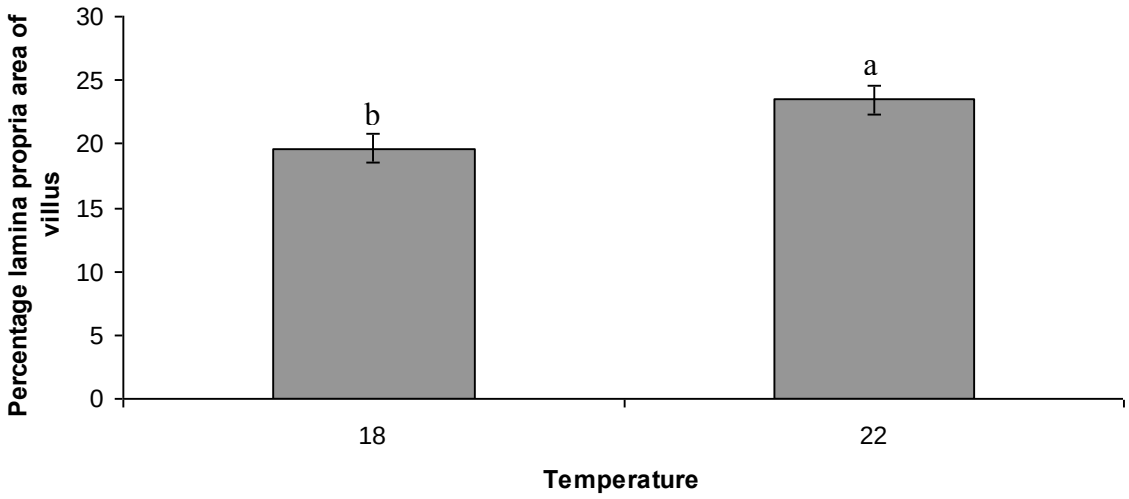

Figure 5 Percentage of lamina propria in relation to villus area at 18ºC and 22 ºC. Means with different superscripts were significantly different (n= 8 replicate tanks).

Mucus layer

The mucus layer in the hindgut of Yellowtail kingfish was thin and discontinuous, occurring predominately between villi, with the tips of the villi not always covered with mucus (Fig. 6).

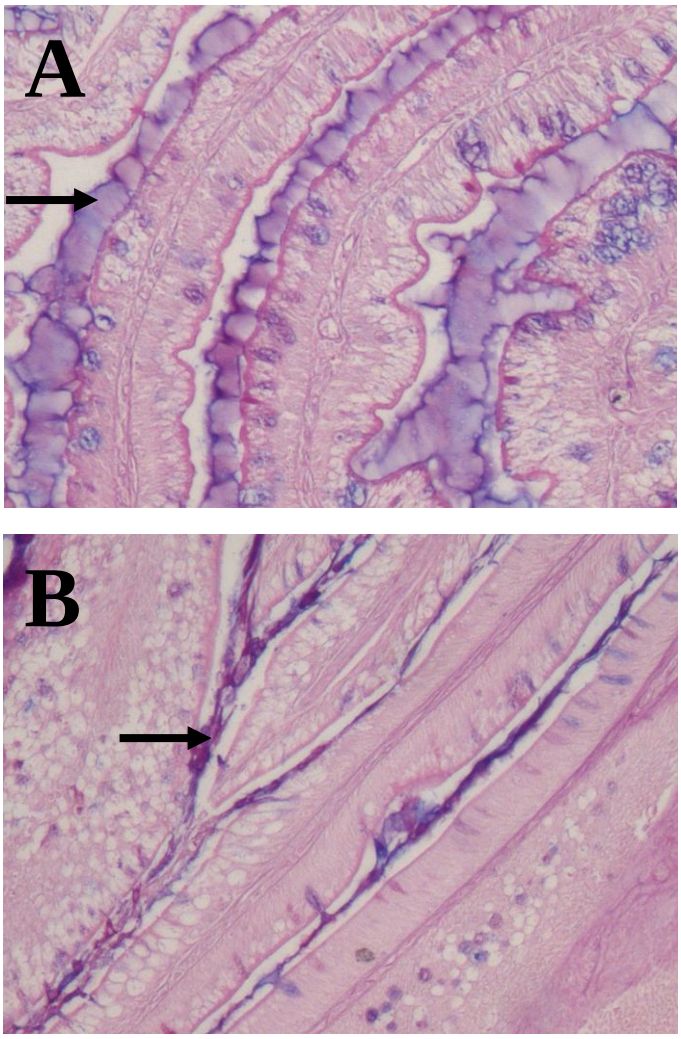

Figure 6 A) Mucus layer (arrow) in the hindgut of Yellowtail kingfish fed 0% SE SBM at 22ºC (100X). B) Mucus layer (arrow) in the hindgut of Yellowtail kingfish fed 30% SE SBM at 22ºC (100X).

Dietary inclusion level of SE SBM was the only factor that significantly affected mucus layer thickness. A significant reduction of mucus layer thickness occurred when fish were fed increasing dietary inclusion of SE SBM. Fish fed the control fish meal diet had a significantly thicker mucus layer than fish fed a diet containing SE SBM (Fig. 7). There was

no significant difference in mucus layer thickness between fish fed 10% SE SBM and fish fed 20% SE SBM. However, the mucus layer was significantly thinner than in fish fed the control fish meal diet. Fish fed 30% SE SBM had a significantly thinner mucus layer compared to fish fed lower dietary inclusion levels of SE SBM.

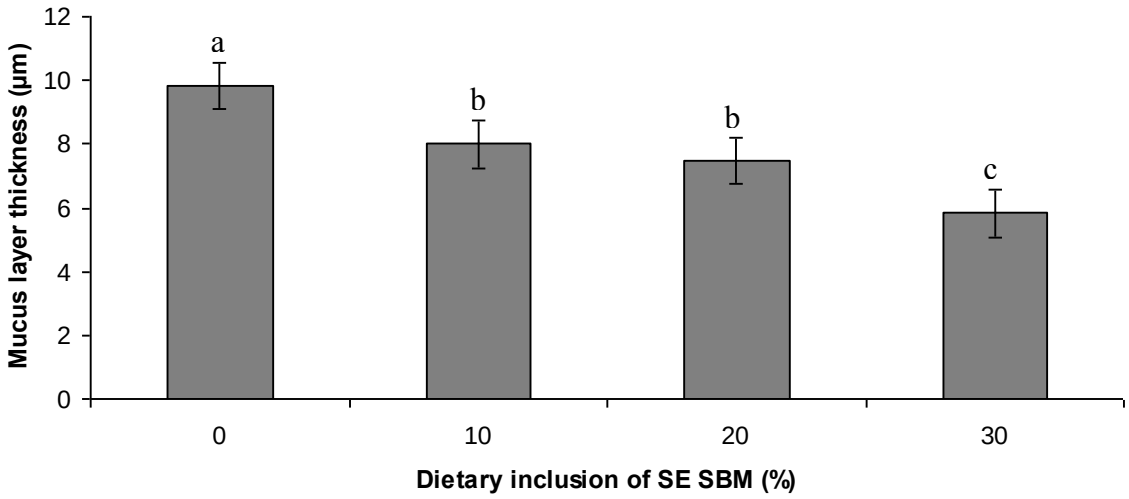

Figure 7 Average mucus layer thickness between villi in the hindgut of Yellowtail kingfish with increasing dietary inclusion of SE SBM. Means with different superscripts were significantly different (n= 4 replicate tanks).

Goblet Cells

Dietary inclusion levels of SE SBM, when fed to fish kept at 22ºC, had no significant effect on total goblet cell number (Fig. 8). No significant difference in goblet cell number was also observed for fish in 18ºC water fed a diet containing 0% and 10% SE SBM compared to fish fed the same diet at 22ºC. However, at 18ºC, a significant increase in goblet cell number was observed when fish were fed a diet containing 20% and 30% SE SBM.

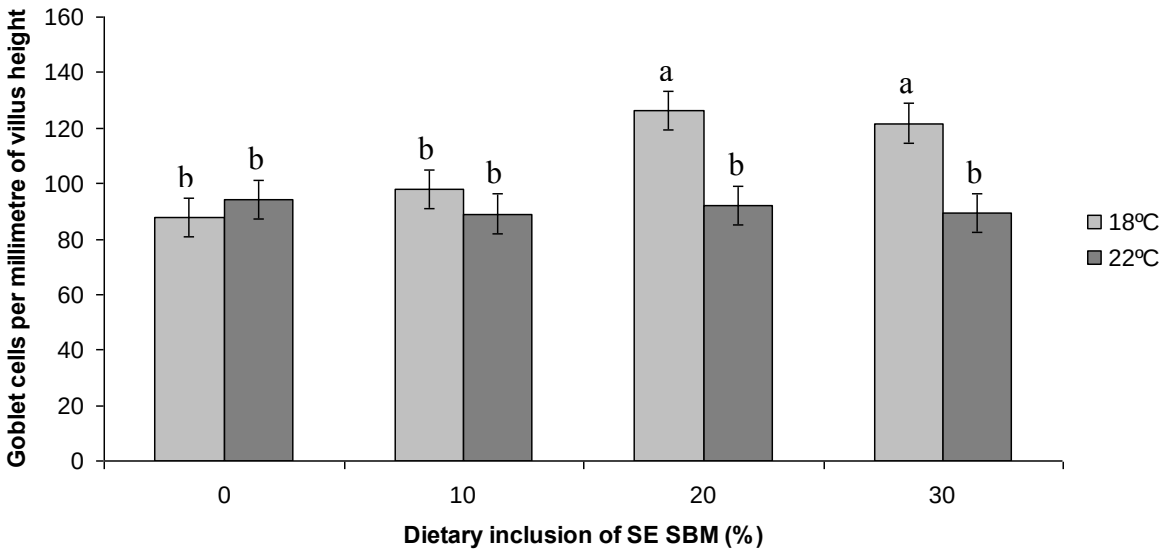

Figure 8 Number of goblet cells per millimetre of villus height in the hindgut of Yellowtail kingfish with increasing dietary inclusion of SE SBM at 18ºC and 22 ºC. Means with different superscripts were significantly different (n= 2 replicate tanks).

Mucin Composition

The interaction between water temperature and total number of goblet cells had a significant effect on the number of goblet cells containing neutral mucins (Fig. 9). A similar number of goblet cells containing neutral mucins were observed in villi with a low number of goblet cells from fish from either water temperature. However, as the number of total goblet cells increased on the villi, there was a more profound increase in goblet cells containing neutral mucins in fish in 18ºC water than in fish at 22ºC.

The interaction between water temperature and total number of goblet cells had a significant effect on the number of goblet cells containing acidic mucins (Fig. 10). However, the effect water temperature had on the number of goblet cells containing acidic mucins was in contrast to neutral mucins. When villi were low in goblet cell number, similar values were observed in fish exposed to either water temperature. When a higher number of goblet cells were counted in the villi, a higher number of goblet cells containing acidic mucins occurred in fish in 22ºC water compared to fish at 18ºC.

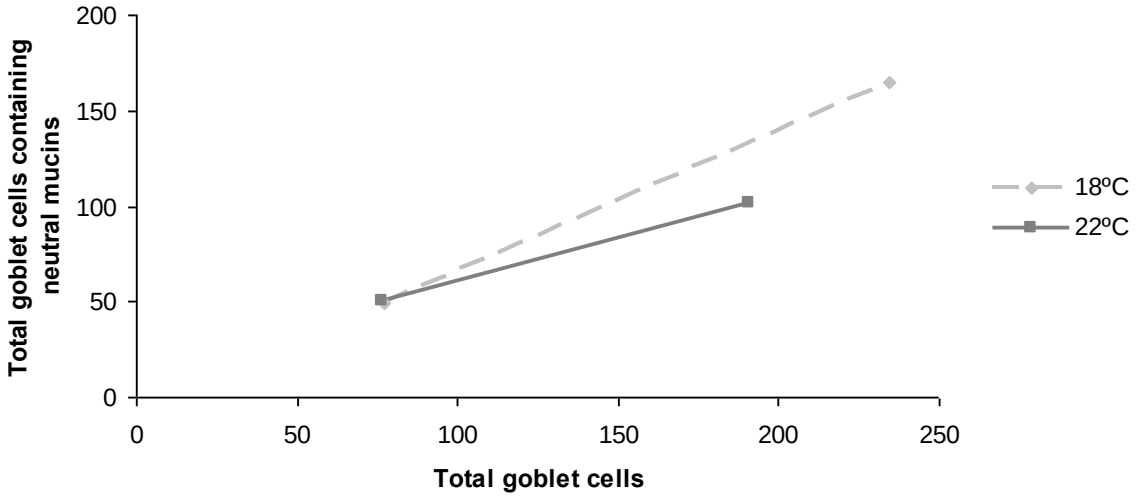

Figure 9 Total number of goblet cells containing neutral mucins in relation to total number of goblet cells per villi at 18ºC and 22ºC (n= 8 replicate tanks).

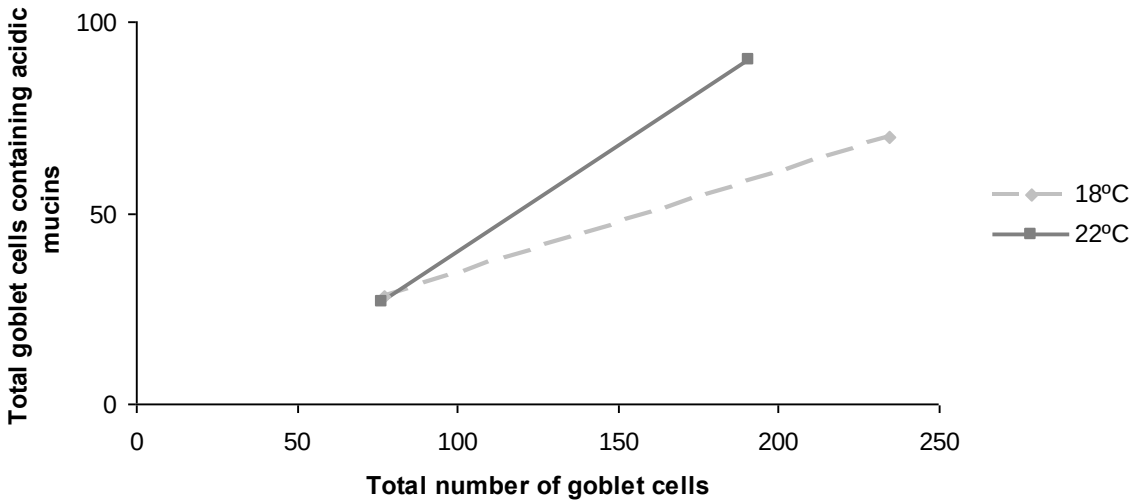

Figure 10 Total number of goblet cells containing acidic mucins in relation to total number of goblet cells per villi at 18ºC and 22ºC (n= 8 replicate tanks)

Discussion

Sub-acute enteritis is a complex syndrome; the mechanisms behind the onset of this condition in Yellowtail kingfish are still debated. Previously, factors suggested to play a role in the onset of sub-acute enteritis in Yellowtail kingfish include high fat feeds, invasion of opportunistic bacteria, cool water temperature and dietary inclusions of plant proteins (Sheppard, 2004). Sub-acute enteritis has a low morbidity rate, exhibiting very few, if any, external symptoms; however, the high mortality rate associated with this condition is a major concern to the Yellowtail kingfish industry (Sheppard, 2004).

The primary aim of this study was to investigate and evaluate the efficacy of the intestinal mucus layer in Yellowtail kingfish through changes in mucin synthesis and secretion associated with increasing dietary inclusion levels of SE SBM at optimal and sub-optimal water temperatures. Water temperature and dietary inclusion level of SE SBM had a significant effect on the final weight gain of Yellowtail kingfish. Fish fed high levels of SE SBM had a significantly thinner mucus layer in the hindgut than fish fed lower levels of SE SBM. Intestinal mucin composition in Yellowtail kingfish was affected by water temperature; a significantly increased synthesis of neutral mucins occurred at 18ºC. In contrast, a significant increase of acidic mucins were synthesised in fish at 22ºC. However, at both water temperatures there were higher levels of neutral mucins synthesised compared to acidic mucins. Changes to the mucus layer thickness and mucin composition may affect the efficacy of the mucus layer to maintain its function, leading to a reduction in the protection of the underlying intestinal mucosa. The reduced efficacy of the mucus layer as a protective barrier may have been one of the factors increasing the risk of sub-acute enteritis developing in Yellowtail kingfish.

The greater effect of water temperature on the final weight gain of Yellowtail kingfish was similarly observed in Australian snapper (*Pagrus auratus,* Sparidae) (Quartararo *et al.*,

1998). Australian snapper were housed in ambient water temperature (ranging from 13 to 18°C), or water that on average was 4ºC higher, and fed either a 64% fish meal diet or a 30% fish meal and 20% SE SBM diet. The weight gain of Australian snapper in the warmer water was greater than double that of fish held at ambient water temperature. However, fish fed the control fish meal diet had a statistically similar weight gain as fish fed the 20% dietary inclusion of SE SBM (Quartararo *et al.*, 1998).

Optimal metabolic function of Yellowtail kingfish occurs at water temperatures between 20 and 25°C with an asymptote at 22.8°C (Pirozzi and Booth, 2009). The link between optimal metabolic function and the temperature dependency of digestive enzymatic function is a possible explanation for the higher weight gain observed at optimal water temperature (22ºC) in this study (Hochachka and Somero, 2002).

The lower weight gain observed in fish fed higher dietary inclusion levels of SE SBM may have occurred for a number of reasons. All experimental diets were balanced for digestible protein and digestible lipid; however, the diets were not balanced on a digestible energy basis. Additional samples were collected to measure the nutrient and energy digestibility coefficients of the feed for Yellowtail kingfish. However, the digestibility of the diets has not been determined yet. Atlantic salmon have considerably lower apparent digestibility coefficients for energy when fed a diet containing extracted and toasted soybean meal compared to a fish meal (Storebakken *et al.*, 1998). Lower energy digestibility of SE SBM compared to fish meal may be one of the potential causes of the reduced weight gain observed at high dietary inclusion levels of SE SBM for Yellowtail kingfish in this study.

Dietary inclusions of SE SBM have been shown in other fish species, such as Atlantic salmon, to cause damage to the intestinal mucosa (van den Ingh *et al.*, 1991, Baeverfjord and Krogdahl, 1996, Urán, 2008), and microvilli (Merrifield *et al.*, 2009). In the current study, no damage to the intestinal mucosa was observed from histological evaluation. However, it is unknown as to whether any damage to ultrastructures, such as microvilli, occurred in this

study. If any damage had occurred, it could be speculated that fish would use energy to repair damaged tissue instead of it being utilised for growth (Szabo, 1989).

The reduced weight gain of fish observed at dietary higher inclusion levels of SE SBM may also be caused by increased synthesis of mucin to replenish the reduced mucus layer. A reduced performance has been observed in poultry when fed low crude protein diets, despite dietary inclusions of free essential amino acids (Moran, 2011). Performance was subsequently improved by increasing dietary levels of non-essential amino acids, particularly glycine, serine and proline. Glycine, serine and proline are prominent in mucin released in the intestine, sourcing these non-essential amino acids has been suggested to dominate mucosal maintenance (Moran, 2011). Due to the reduced mucus layer thickness and increased goblet cell numbers observed in Yellowtail kingfish fed high SE SBM inclusion levels, the utilisation of amino acids for mucin synthesis may in turn have affected final weight gain. Additionally, the extra metabolic energy expense associated with the production of the extra goblet cells and mucins in the SE SBM fed fish may have affected growth. These areas will need further research.

The synthesis and degradation of the mucus layer are natural processes; a balance between these processes is required to protect the epithelium against harmful organisms or detrimental compounds present in the diet (Corfield *et al.*, 2001). Bacteria have been hypothesised to play crucial roles in a number of inflammatory diseases including Crohn‘s disease in humans (Schultsz *et al.*, 1999) and necrotic enteritis in poultry (Collier *et al.*, 2008). With certain diseases, such as necrotic enteritis in poultry, *Clostridium perfringens* is not detrimental until the mucosa is compromised by predisposing factors such as *Eimeria* parasites. Inflammation caused by *Eimeria* parasites are believed to allow *C. perfringens* to rapidly replicate and produce toxins (Van Immerseel *et al.*, 2009). An increased secretion of mucin may occur due to the initial exposure to *Eimeria*, which has been speculated to allow

C. perfringens to utilize mucin as a nutritional substrate, providing a growth advantage to the species (Collier *et al.*, 2008).

The survival of pathogens, microbiota composition and the functioning of the mucus layer is determined by the non-absorbed nutrients from the diet (Bovee-Oudenhoven and van der Meer, 2001). In a previous study, no change in viable counts were observed for both autochthonous (native) and allochthonous (foreign) bacteria in the intestine of Rainbow trout (*Oncorhynchus mykiss,* Salmonidae) when fed either a control fish meal diet or a diet with 50% fish meal replaced with SE SBM (Merrifield *et al.*, 2009). However, alterations to the bacterial population did occur. A higher number of *Psychrobater* spp. and yeast while a reduction in *Aeromonas* spp. were observed in fish fed a diet with 50% fish meal replaced with soybean meal compared to fish fed a control fish meal diet (Merrifield *et al.*, 2009). An inclusion of SE SBM fed to Yellowtail kingfish may alter the luminal environment in the intestinal tract, which may disrupt intestinal-immune homeostasis, potentially changing the microbial profile leading to an overgrowth of undesirable bacterial species, and the possible development of sub-acute enteritis. Collecting samples for microbial profiling from the digestive tract of Yellowtail kingfish fed diets containing SE SBM would be required to test this hypothesis.

The principal components influencing the physical and functional properties of the mucus layer are mucins (Forstner and Forstner, 1994). Acidic mucins have a strong negative charge; due to this, differential staining can easily distinguish them from neutral mucins, and thus alterations to mucin composition in goblet cells can be assessed. Bacteria have evolved a number of specific mucin-degrading compounds including glycosidases (Hoskins *et al.*, 1985). The strong negative charge exhibited by sialylated and sulphated mucins increases the resistance to degradation by bacterial enzymes compared to neutral mucins (Parker *et al.*, 1995, Raouf *et al.*, 1992) as well as offering superior binding sites for adhesion molecules (Van Klinken *et al.*, 1995). At 18ºC Yellowtail kingfish had an increased synthesis of neutral

mucins compared to fish at 22ºC. In contrast, at 22ºC Yellowtail kingfish were observed to have an increased synthesis of acidic mucins compared to fish at 18ºC. Yellowtail kingfish may be more susceptible to infection at 18°C due to the reduced synthesis of acidic mucins, if fish are then fed diets containing high levels of SE SBM, the mucus layers protective role may be further reduced, and may exacerbate the risk of infection and the development of sub-acute enteritis.

Mucus layer thickness has previously not been reported in fish; however, regional variation in mucus layer thickness from the stomach to the colon observed in rodent models and humans suggests each region has an optimal thickness for protection of the underlying intestinal mucosa (Atuma *et al.*, 2001). The reduced mucus layer thickness and the change to mucin synthesis observed in this study may increase the vulnerability of underlying epithelial cells to antinutrients present in SE SBM. Alcohol soluble compounds, such as saponins, in soybean meal have been identified as the primary cause of sub-acute enteritis in the hindgut of Atlantic salmon (van den Ingh *et al.*, 1996). Saponins increase the permeability of intestinal mucosal cells, facilitating the uptake of substances not normally absorbed, while inhibiting active transport of substances that are normally absorbed (Johnson *et al.*, 1986). Water temperature significantly affects gut transit time in Yellowtail kingfish. Digesta takes approximately three times longer to be voided at a water temperature of 13ºC compared to 21ºC (Miegel *et al.*, 2010). The reduced gut transit time observed at low water temperatures in conjunction with a compromised mucus layer may increase the duration that the intestinal mucosa is exposed to the antinutrients present in SE SBM.

Typical characteristics of sub-acute enteritis, such as decreased villus height, villus area, increased lamina propria area and increased goblet cell number were measured in this study (van den Ingh *et al.*, 1991, Baeverfjord and Krogdahl, 1996). A number of other characteristics have been semi-quantitatively scored depending on the severity of sub-acute enteritis in Atlantic salmon including supranuclear vacuoles, eosinophilic granulocytes and

sub-epithelial mucosa (Urán, 2008). However, inflammation to the hindgut was not observed in this study, with only mild changes to the intestinal morphology detected, thus scoring these additional parameters would have been ineffective in determining the severity of the condition.

Changes to intestinal morphology in Atlantic salmon fed increasing inclusion levels of SE SBM over 57 days observed no signs of sub-acute enteritis in fish fed control fish meal diet (Urán *et al.*, 2009). Fish fed 20% soybean meal, reached maximum scores for most parameters measured, with a severely inflamed hindgut observed (Urán *et al.*, 2009). Sub-acute enteritis was less severe in fish fed 10% soybean meal than fish fed 20% soybean meal. Despite only mild inflammation occurring in fish fed 10% soybean meal, the increased goblet cell number appeared more responsive than other variables measured and received a high enteritis score. This increased differentiation of goblet cells has been suggested to result in an increased mucus production (Urán, 2009). Data from the current study supports this hypothesis, with a degradation of the mucus layer possibly acting as a stimulus for the differentiation of goblet cell to replenish the mucus layer. The increased synthesis of mucin may be crucial in protecting the intestinal mucosa, indicating that the initial increase in goblet cell number observed in this study may be a first stage of sub-acute enteritis.

In this study, the lamina propria in fish was significantly greater at 22ºC than at 18ºC. Despite this, the lamina propria in all fish in this study appeared much smaller than lamina propria in Atlantic salmon with fully developed sub-acute enteritis (Urán, 2008). The increased lamina propria size in fish at 22ºC may have been due to improved gut development occurring in fish at optimal water temperatures.

Dietary inclusion level of SE SBM and water temperature had no significant effect on villus height or villus area in Yellowtail kingfish. Villi morphology appeared similar to Atlantic salmon when fed a control fish meal diet (Urán, 2008). However, the possible first stage of sub-acute enteritis observed in this study indicates the mucosa had been

compromised. This study suggests Yellowtail kingfish are more tolerant to dietary inclusions of soybean meal than Atlantic salmon (Urán, 2008), but less tolerant than species adapted to plant proteins, such as Channel catfish (Evans *et al.*, 2005). It would be interesting to assess changes to intestinal morphology and if sub-acute enteritis is induced in Yellowtail kingfish reared in colder waters indicative of those experienced during winter months when fed additional dietary inclusions of SE SBM.

In conclusion, due to the substantial increase in price and reduced availability of fish meal aquaculture companies are turning to more economic and ecologically sustainable protein sources. Soybean meal is an alternate protein source of interest due to its relatively high protein content, low cost, and availability. However, this study demonstrates that feeding high dietary inclusion levels of SE SBM to Yellowtail kingfish results in a reduced growth rate and protective efficacy of the mucus layer, inturn compromising the gastrointestinal tract. If Yellowtail kingfish are fed a diet containing SE SBM and are stressed further, such as exposure to colder water temperate or fed for a longer duration, sub-acute enteritis may subsequently develop. Based on the growth performance and the reduction observed in the mucus layer thickness, increase in goblet cell number and changes in mucin composition, SE SBM inclusion levels in the diet for Yellowtail kingfish of this size range should be restricted to 10% at 18°C and 20% at 22°C.

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Appendices

Appendix 1: Fixation Method Development

Due to the mucus layer not observed in samples fixed in Carnoy‘s solution (60% ethanol, 30% chloroform and 10% acetic acid) collected in a method development conducted in August 2010, a second method to preserve the mucus layer was used. The tissue was opened longitudinally in a $1cm^{-3}$ envelope of pig‘s liver, wrapped in aluminium foil and snap frozen in liquid nitrogen where it was transferred to a -80ºC freezer and stored until sectioned (Jordan *et al.*, 1998). However, in this current study, the mucus layer in the distal intestine of Yellowtail kingfish was apparent and was observed greatest in tissue fixed in Carnoy‘s solution compared to samples opened on pig liver.

Appendix 2: Staining Protocol

Haematoxylin and Eosin

60ºC Oven	10 mins
Histolene	5 mins
100% ethanol	2 mins
80% ethanol	2 mins
30% ethanol	2 mins
Lille Mayer‘s Haematoxylin	5 mins
Tap water	20 secs
Acid ethanol	7-10 secs
Running tap water	10 mins
Eosin	10 secs
80% ethanol	7 secs
100% ethanol	2 mins
Histolene	5 mins

Periodic Acid Shiffs/Alcian Blue pH 2.5

Histolene	10 mins
100% ethanol	2 mins
80% ethanol	2 mins
30% ethanol	2 mins
RO water	2 mins
Alcian Blue pH=2.5	30 mins
3% acetic acid	2 dips
Running tap water	5 mins
RO water	1-2 mins
Periodic acid	20 mins
Running tap water	3 mins

Schiff‘s Reagent	20 mins
Running tap water	3 mins
70% ethanol	1 min
80% ethanol	1 min
100% ethanol	2 mins
Histolene	5 mins

High Iron Diamine/Alcian Blue pH 2.5

Histolene	10 mins
100% ethanol	2 mins
80% ethanol	2 mins
30% ethanol	2 mins
RO water	2 mins
HID solution*	16 hours
Running tap water	3 mins
Alcian Blue pH=2.5	5mins
Running tap water	3 mins
70% ethanol	1 min
80% ethanol	1 min
100% ethanol	2 mins
Histolene	5 mins

HID solution*

360mg N, N – dimethyl-meta-phenylenediamine dihydrochloride

60mg N, N – dimethyl-para-phenylenediamine dihydrochloride

142.2ml RO water

6ml 42% ferric chloride