FEED ATTRACTANTS FOR JUVENILE CHINOOK SALMON (*Oncorhynchus tshawytscha*) PREPARED FROM HYDROLYSATES OF PACIFIC HAKE (*Merluccius productus*)

by

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ABSTRACT

The incorporation of soybean meal in aquaculture diets can lower feed ingredient costs, but also produces organoleptically unfavorable conditions for several salmon species. Addition of feed attractants could mask the undesirable tastes associated with soybean meal, enhance palatability of the diet, and increase feed intake. The objective of this study was to assess the feasibility of utilizing hydrolysates from Pacific hake as feed attractants in the diet of juvenile Chinook salmon. A 5-week palatability feeding trial was conducted to assess hydrolysates produced by Alcalase or Flavorzyme proteolysis of Pacific hake, compared to commercial feed attractants (krill meal and screen 1 & 2), as feed attractants in diets containing 20% soybean meal. These were compared with diets having no added feed attractants (negative control) and no soybean meal (positive control). All of the fish fed the soy-based diets had significantly ($p<0.05$) lower growth parameters when compared with fish fed the fishmeal based positive control diet. However, addition of Alcalase hydrolysate, krill or screen 1 provided increases in fish weight gain, feed efficiency ratio, and specific growth rate ($p<0.05$) compared with fish fed the negative control soy-based diet. The percent of feed dispensed that was not consumed by the fish was lowered ($p<0.05$) with the use of these three aforementioned attractants when compared to fish fed the negative control diet. Increased daily and total feed intake were also observed for the fish fed the Screen 1 coated diets ($p<0.05$). Fish fed Alcalase or Flavorzyme hydrolysate containing diets were tested for the presence of *Kudoa* spores, which were not detected in any of the fish tested. This study demonstrated the potential for Pacific hake, an underused marine source, to be converted to a feed attractant to help minimize the problems associated with dietary use of soybean meal in salmon aquaculture.

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1.0 INTRODUCTION

The rapid increase in the global population has created a strain on the world food supply. Among these foods, fish and fish-based products are in very high demand because of their nutritional quality and health benefits (FAO-FD, 2006). Fish contain high quality protein and are rich sources of polyunsaturated fatty acids, especially omega-3 fatty acids such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. However, due to the fishing and consumption rates exceeding the breeding and regenerating properties of certain wild fish stocks, the oceans are being gradually depleted of these healthy food sources (FAO-FD, 2006). One possible solution, which has been in development for several decades, is the use of aquaculture and farmed fish to help meet the growing food demands and maintain overall sustainability.

Over the years, the demand for fish products has been increasing, while the prices for fishmeal have been constantly fluctuating. Fishmeal diets, which are based heavily on marine proteins, can be limiting and costly. For continued growth, the industry must reduce its use of fishmeal, one of the most expensive ingredients in fish diet formulations, in favor of cost efficient and sustainable alternative protein sources (Oikawa and March, 1997). Therefore, fish farmers have been looking for less expensive alternative protein sources for use as fish feed ingredients in aquaculture. These alternative protein sources are generally derived from terrestrial animal or plant proteins, such as poultry byproduct meal, canola meal, and soybean meal (Gatlin *et al*., 2007). Soybean meal, which has been used in aquaculture diets to help reduce feed ingredient costs, is a highly favored protein source due to its high quality protein content and amino acid profile compared with other plant proteins (Gatlin *et al*., 2007). However, the dietary inclusion of these foreign proteins, such as soybean meal can produce unfavorable palatability conditions for several finfish species, especially Chinook salmon (Bureau *et al*., 1998). The use of dietary

feed attractants, made from marine sources, when applied onto the feed may mask the undesirable properties found in soybean meal and enhance the palatability of the diet.

One underutilized species of fish, is the Pacific hake (*Merluccius productus*), which is found off the West coast of Canada and the United States of America. Pacific hake are highly undervalued because of poor postmortem flesh texture. *Kudoa paniformis*, a myxozoan parasite found in Pacific hake begins to deteriorate the muscle tissues of the fish after it is deceased, resulting in the soft fillet consistency (Kabata and Whitaker, 1981). Extensive research has been conducted with the goal of turning this low economically valued fish into a higher quality marine bioproduct. Some of the possibilities include rich, oil-based fertilizers, oligopeptides with nutraceutical functions, or smaller peptides with palatability enhancing properties for other carnivorous fish (Baek and Cadwallader, 1995). In this study, the focus was to explore the potential for using low concentrates of hydrolyzed Pacific hake protein as feed attractants for juvenile Chinook salmon fed diets based on soybean meal as a major protein source.

2.0 LITERATURE REVIEW

2.1 Aquaculture

The aquaculture industry is the fastest growing food production sector in the world. It has increased from 3.9% in total food production weight in 1970 to 33% in 2005 (FAO-FD, 2006). This means the compounded rate on average for aquaculture has been 9.2% annually since 1970, which is significantly higher than the 1.4% for wild caught fish and the 2.8% for terrestrial farmed animal production (FAO-FD, 2006). Presently, over 33% of the fish consumed in the world is farmed (Pickova and Morkore, 2007). The aquaculture industry has been growing in response to this high demand for farmed fish products, while the worldwide prices and stocks for fishmeal have respectively increased and remained relatively stable. Thus, these fishmeal based diets have become more expensive to produce with the limiting fishmeal supply. Moreover, the salmon aquaculture industry has also undergone a transition towards diets with high energy and lipid content with reduced concentrations of carbohydrates (Azevedo *et al*., 2004). In the past 20 years, dietary lipid content has increased from approximately 12% to 40% with dietary carbohydrates decreasing from approximately 40% to 15% (Azevedo *et al*., 2004). This has placed greater stress on the already limiting stocks of fishmeal since fishmeal has a higher lipid and lower carbohydrate content than most other ingredients, such as the plant based raw materials. Fishmeal is still used at an inclusion level greater than 50% in certain aquaculture diets (Glencross *et al*., 2007). Clearly, these diets are much too reliant on the global fishmeal supply. For continued growth, the industry must reduce its use of fishmeal, one of the most expensive ingredients in fish diet formulations, and instead use more cost efficient and sustainable alternative protein sources of plant and animal origin.

2.2 Palatability

Plant and terrestrial animal derived food protein products are foreign substances to fish and therefore, can produce unfavorable palatability conditions for several finfish species. This change in taste could potentially decrease the palatability of the diet for the fish. Palatability is defined as how acceptable the taste of a diet is and how tolerable it is compared to the flavors that the host is familiar with (Glencross *et al*., 2007). More specifically, diet palatability can be defined by the combined effects of how attractive the feed is to the fish and the quantity of feed the fish ingests (Glencross *et al*., 2007). The easiest method to measure the degree of palatability would be to quantify the amount of test feed consumed by a fish species and then relate this amount to the amount of a conventional control diet that is consumed under identical conditions. Palatability helps a species to reach satiety since fish will generally not stop eating organoleptically acceptable diets until they have met their daily energy needs (Glencross *et al*., 2007). In order for the measurement of palatability to be accurate and a difference in feed intake detected, fish must be given the chance to refuse the feed and thus fish must be fed past their apparent satiation stage (Glencross *et al*., 2007).

2.3 Alternative feed ingredients

Fish farmers have been looking for less expensive alternative sources of proteins for fish feed ingredients. These alternative protein sources have been derived from terrestrial animals, algae, bacteria, fungi, plant proteins, or genetically modified plant proteins (Pickova and Morkore, 2007). Many plant-based proteins, such as cereal grains, oilseeds, and legumes have been tested as protein and energy concentrates in other food sectors (Gatlin *et al*., 2007). A feedstuff, to be successful, must mimic the characteristics of fishmeal as much as possible. Fishmeal is known for its well balanced amino acid profile, high protein content, wide availability, good nutrient

digestibility and lack of antinutrients (Gatlin *et al*., 2007). These alternative feed ingredients not only have to mirror all these characteristics, but they should have a comparable or lower price as well as minimal handling, shipping, and storage requirements compared to fishmeal (Gatlin *et al*., 2007). Other beneficial characteristics include having a minimal environmental impact, producing acceptable fish flesh quality, providing sufficient palatability and a positive effect on fish growth and health (Gatlin *et al*., 2007). The aquaculture industry would like to use ingredients that are very high in energy to help reduce nitrogen waste production, increase feed efficiency and reduce the amount of feed fed to fish (Azevedo *et al*., 2004).

Fish require certain nutrients, such as lecithin and phosphatidylcholine that are not required by terrestrial animals and those nutrient requirements should be taken into consideration when looking for alternative feed ingredients (Brown *et al*., 1997). Although essential fatty acids are required to maintain proper fish health, fatty acids are not considered when selecting dry feed ingredients because additional fish oils are generally added to the diets to help correct for any differences in lipid fatty acid composition.

Some plant ingredients encompass the majority of these characteristics, but some, like wheat gluten meal and soy protein concentrate are too expensive to produce compared to fishmeal (Gatlin *et al*., 2007). Therefore, more affordable alternative ingredients would have to be used in conjunction with additional amino acid supplements and flavorings to make those alternative protein concentrates effective as fishmeal replacements. Specific alternative ingredients being tested and used in aquaculture are poultry byproduct meal, blood meal, feather meal, soybean meal, whole wheat, cottonseed meal, barley meal, wheat flour, pea, corn gluten meal, lupins, and canola meal and concentrate (Gatlin *et al*., 2007). Since the dietary requirements of fish are different from those of terrestrial animals, it is important that these protein sources are processed

correctly so that essential nutrients are not lost in the process and their nutritional composition is adequate for fish health. In addition, any bioactive compounds that could potentially be detrimental to fish health or act as taste deterrents should be minimized or removed from the feedstuff. This includes the reduction of antinutrients, fibers, starch, and non-soluble carbohydrates that can negatively affect fish health (Gatlin *et al*., 2007). However, challenges remain as reduced growth performance and feed efficiency are frequently associated with the use of foreign proteins (Aksnes *et al*., 2006a). Poor performance can occur, even when nutrient levels in the diets are well balanced and the growth inhibitors and antinutrients in the diet are minimized (Aksnes *et al*., 2006a).

2.3.1 Soybean meal

Soybean, *Glycine max* Linnaeus, is the most abundant oilseed crop in the world (Brown *et al*., 1997). Soybeans are versatile and have a broad spectrum of uses in the food industry. A large fraction of the soybean production goes towards the production of soybean oil, which is extracted from the oilseed (NRC, 1993). This results in a dry cake like material that has a very high protein quality and can be processed further to yield various soybean products (NRC, 1993). Products studied for potential use in fish diets include soybean meal, soy flour, soy protein isolate and soy protein concentrate (NRC, 1993). The predominant soy product used in aquaculture is soybean meal, either with hulls (44% crude protein dwb) or in a dehulled state (48% crude protein dwb) (NRC, 1993).

Soybean meal is inexpensive to produce, widely available because of high global production and thus the predominant plant protein based ingredient used in aquaculture. It is an excellent protein source due to its high crude protein content, high quality protein and sufficiently balanced amino

acid profile (Gatlin *et al*., 2007). Soybean meal, however, is not without flaws and these limitations must be addressed before it is included in fish diets.

2.3.1.1 Soybean meal problems

Despite having a superior amino acid profile compared to other plant based ingredients, soybean meal is still inferior to fishmeal. All ten essential amino acids and tyrosine are present at much lower concentrations in soybean meal when compared to fishmeal (Gatlin, *et al*., 2007). The most limiting amino acids in soybean meal that usually require additional supplementation are lysine and the sulphur amino acids cysteine and methionine (Brown *et al*., 1997). This problem can be overcome with the use of amino acid supplementation in the diet. Other soy products, like soy protein isolate and soy protein concentrate, contain higher essential amino acid contents, but these products tend to be very expensive to produce and therefore are not economically feasible for aquaculture. Soybean meal is lower in crude fat, vitamins, and ash than fishmeal (Gatlin *et al*., 2007). However, these problems can be easily solved by supplementing diets with oil, vitamins, and minerals. Soybean meal has a high carbohydrate content in the form of oligosaccharides including stachyose and raffinose, which are indigestible because fish lack the enzymes required for their hydrolysis (Refstie *et al*., 1998). These complex oligosaccharides will increase the viscosity of chyme in the gastrointestinal tract and hinder the absorption of other essential nutrients, such as certain fats and minerals (Refstie *et al*., 1998). This, in turn, can lead to decreased growth performance, feed efficiency and eventually enteritis (Refstie *et al*., 2005). One possible solution would be to hydrolyze the oligosaccharides in the soybean meal through bacterial or fungal fermentation (Refstie *et al*., 2005) to help break down and reduce the carbohydrate levels in the soybean meal (Wu *et al*., 2003). Heat treatment during the extrusion of diets also helps to improve the bioavailability and digestibility of the carbohydrate portion of the soybean meal by gelatinizing the starch (Goda *et al*., 2007b).

2.3.1.2 Soybean meal antinutrients

Several antinutrients in soybean meal cause fish health problems. Phytic acid in the soybean meal can bind cationic minerals, especially zinc, rendering them unavailable to the fish, mostly salmon and trout (Gatlin *et al*., 2007). Phosphorus is stored in soybean meal in the form of phytate (4% in soybean meal), which has very low bioavailability in the fish digestive tract (Gatlin *et al*., 2007). Phytic acid can only be partially destroyed by heat processing and its dephosphorylation can only be achieved to a certain extent or completed by adding the enzyme phytase to feeds or through pretreatment of the soybean meal itself with phytase (Gatlin *et al*., 2007). This, in turn, increases the bioavailablity of phosphorus and other minerals. Another antinutrient is lectin, which can decrease nutrient absorption, increase pancreatic secretions, decrease intestinal enzyme activity, and cause hyperplasia and hypertrophy (Gatlin *et al*., 2007). In contrast to phytic acid, lectins cannot be destroyed by enzymes, but can be inactivated when heated above 60ºC and destroyed when heated to 100ºC for 5 minutes (Gatlin *et al*., 2007). This is generally not a problem because the majority of fish feeds are extruded at 90 to 100ºC or even higher, which provides sufficient heat and time to inactivate the lectins in the soybean meal. Soybean meal also contains several protease inhibitors that can have detrimental effects on fish growth and health (Drackley, 2000). These inhibitors can usually be inactivated with the use of adequate heat treatments. However, extreme heat treatments can also decrease the protein quality of the soybean meal through oxidation and unwanted fusion to other compounds (Drackley, 2000). In efforts to maintain sufficient protein quality, all the protease inhibitors are not inactivated, which explains why soybean meal has a lower availability of nutrients than fishmeal (Drackley, 2000).

Soybean meal also contains saponins, which are steroid glycosides that vary in structure (Bureau *et al*., 1998). The class of saponins found in soybean meal is soyasaponins and there are considerable amounts of these compounds in soybeans and other legumes (Bureau *et al*., 1998). Soyasaponins decrease feed intake and induce enteritis in certain fish species (Bureau *et al*., 1998). The amount of soyasaponins found in soybean protein products is dependent on the methodology used in processing and the type of product produced. For example, soy protein isolates extracted with alcohol contain very little soyasaponins because alcohol helps break the bond between the saponin and the protein (Bureau *et al*., 1998). However, water does not remove a lot of the saponins from soybean meal even though saponins have good water solubility (Bureau *et al*., 1998). Overall, saponins in fish feed are problematic because unlike some other antinutrients, saponins cannot be inactivated or removed by heat processing (Zhou *et al*., 2005).

Soy protein concentrate and soy protein isolate have undergone processing to remove or deactivate oligosaccharides, fibers, and antinutrients (Brown *et al*., 1997). These ethanol extracted products have been tested in fish diets and they contributed to increased growth rate when compared to regular soybean meal (Murai *et al*., 1989; Shimeno *et al*., 1992; Olli *et al*., 1994; Kaushik *et al*., 1995). However due to the intensive processing involved, the cost of soy protein concentrate can exceed that of high quality fishmeal and fish protein hydrolysates (Bureau *et al*., 1998). Therefore, those soy protein concentrates would not be economically practical for use on a large scale in aquaculture (Bureau *et al*., 1998).

2.3.2 Alternative ingredients in salmonid aquaculture

Many studies have tested less expensive alternative ingredients as dietary ingredients for salmonid species, the most globally farmed fish. Atlantic salmon is the most extensively farmed salmonid species, followed by Chinook salmon, rainbow trout, and Coho salmon (Kent, 2000).

One study evaluated soy protein concentrate as a fish feed ingredient for Atlantic and Coho salmon. The fish displayed significantly lower weight gains and feed conversion ratios when they were fed a diet with a 30% inclusion rate of this protein product by replacement of fishmeal compared to the fish fed the control diet without soy protein concentrate (Brown *et al*., 1997). Similar results were found when all the fishmeal in the diets for Atlantic salmon was replaced with a combination of wheat gluten and corn gluten meal (Espe *et al*., 2006). The fish fed the plant-based diets had lower feed intake, fat retention and growth rates than the fish fed the fishmeal control diet (Espe *et al*., 2006). The use of potato starch as a non-protein energy source in diets for juvenile Atlantic salmon led to comparable negative effects with lower feed utilization compared to the fish fed the fishmeal control diet (Hemre *et al*., 2000). In comparison, Mundheim *et al*. (2004) performed an experiment with Atlantic salmon fed different combinations of dietary plant proteins, including wheat gluten meal, corn gluten meal and soybean meal. Consistent with the aforementioned studies, there was a positive correlation between the amount of fishmeal in the diet and feed efficiency, fish growth, and lipid, protein and energy digestibility values (Mundheim *et al*., 2004). However, these parameters did not decrease significantly until plant proteins replaced 50% of the total protein in the diets. There were no significant differences noted between fish fed diets containing 2, 6, and 10% soybean meal. Growth performance of the fish significantly decreased when soybean meal was included at 14% of the diet. The digestibility of the majority of amino acids was also reduced in fish fed

plant protein diets compared to fish fed the fishmeal control diet (Mundheim *et al*., 2004). This strengthens the idea that when plant proteins are included in the diet at a high enough level, there will be a negative impact on fish growth and performance.

There have been negative effects in Atlantic salmon performance when feather meal was used as a fishmeal protein replacement (Bransden *et al*., 2001). However, Atlantic salmon did not exhibit a decrease in weight gain or any negative growth effects as long as feather meal or dehulled lupin meal did not replace more than 40% of the protein in the diets (Bransden *et al*., 2001). Therefore, Atlantic salmon displayed some degree of tolerance for plant and terrestrial animal-based proteins. This tolerance was also reported in another Atlantic salmon study where 33% of the fishmeal was replaced with soybean meal without any adverse effects (Carter and Hauler, 2000). Furthermore, this tolerance for plant and terrestrial animal-based proteins was also seen in a study done by Refstie *et al*. (1998), where Atlantic salmon fed a fishmeal control diet were compared to those fed diets containing either soybean meal (33.9% of the total diet) or soybean meal with reduced antinutrients and oligosaccharides (28.1% of the total diet), with both soybean protein products included at 40% of the total protein in the diet. During the first 28 days, fish fed the regular soybean meal diet exhibited a longer acclimation period, decreased growth, decreased feed conversions, and lower fat, nitrogen and energy digestibility values than the salmon fed the fishmeal control diet (Refstie *et al*., 1998). However, the daily feed consumption during the last 27 days of the 55-day study was similar for all of the fish fed diets, indicating that Atlantic salmon can eventually adapt to some of the antinutrients found in soybean meal (Refstie *et al*., 1998). The Atlantic salmon also tolerated and accepted the diet containing the soybean meal with reduced antinutrients and oligosaccharides very well and exhibited similar growth rate to the fish fed the fishmeal control diet (Refstie *et al*., 1998).

One experiment looked at using various lupin and soybean protein based products, included in the diet at 30% of the protein, for Atlantic salmon and rainbow trout (Glencross *et al*., 2004). Soybean protein concentrate in the diet gave results similar to the study done by Brown *et al*. (1997). The fish fed the soybean protein concentrate diets displayed significantly lower energy digestibility values when compared with the fish fed the fishmeal control diet. Also, the Atlantic salmon in the study exhibited a better tolerance to the reduction of fishmeal in the diet than the rainbow trout (Glencross *et al*., 2004).

Fish health is not the only problem associated with the use of plant and terrestrial animal based proteins as feed ingredients. The sensory quality of certain fish, such as rainbow trout, can also be altered when the diet formulations are changed (de Francesco *et al*., 2004). For example, a high level of plant based ingredients used in the feed can give a milder fish taste to the flesh and using less fishmeal, containing natural pigmentation, in the diets can give fish a lighter colored flesh (de Francesco *et al*., 2004).

Some species of fish cannot tolerate soybean protein products in their diets because of issues related to antinutrients, and Chinook salmon are the most sensitive salmonid species with respect to the dietary inclusion of these types of products (Bureau *et al*., 1998). Juvenile Chinook salmon seem to be the most sensitive, whereas other species like Atlantic salmon, Coho salmon, and rainbow trout are far more tolerant (Bureau *et al*., 1998). Two studies were performed on Chinook salmon and rainbow trout to test their feed intake, intestinal mucosa, and growth when fed diets with soy protein isolates and alcohol extracts from soybean (Bureau *et al*., 1998). Negative effects on Chinook salmon health and growth were strongly due to the presence of soyasaponins in the soybean containing diets (Bureau *et al*., 1998). However, other antinutrients could not be ruled out as contributors to the negative effects demonstrated by the Chinook

salmon fed the soybean products (Bureau *et al*., 1998). The purified alcohol extracts from soybean meal, containing a higher concentration of saponins than soybean meal, acted as feeding deterrents and negatively affected growth performance and intestinal mucosa when included in the diets for Chinook salmon (Bureau *et al*., 1998). Another possible cause for the poor performance of salmonids fed diets with plant-based proteins is the reduced digestibility of the diets due to the presence of growth inhibitors (Ollii *et al*., 1995).

2.3.3 Alternative ingredients in other fish aquaculture

Salmonids are not the only fish cultured in aquaculture operations or the only types of fish affected by the use of plant and terrestrial animal based dietary ingredients. For example, a high inclusion of soybean meal in the diet of Atlantic cod caused a reduction in protein retention, feed efficiency ratio, and amino acid and lipid digestibilities (Refstie *et al*., 2006). Soybean meal can replace up to 20% of the dietary protein in Atlantic cod diets without any negative effects on growth performance (Aksnes *et al*., 2006b). An acceptable level of dehulled soybean meal in the diet that did not cause any significant negative effects was found in an experiment on olive flounder (Choi *et al*., 2004). In this regard, dehulled soybean meal could replace up to 30% of the fishmeal if it was supplemented with amino acids and a feed attractant whereas dehulled soybean meal alone was unable to replace up to 20% of the fishmeal. Any inclusion level higher than this resulted in a significant decrease in weight gain and feed efficiency ratio of the flounder (Choi *et al*., 2004).

Tolerance levels for soybean protein products have also been found in other species of fish when fishmeal has been replaced, e.g. 20% replacement with soybean meal for yellowtail, 90% for red drum, and 25% replacement with soy protein concentrate for turbot (Shimeno *et al*., 1993; McGoogan and Gatlin III, 1997; Day and Plascencia Gonzalez, 2000). Similarly, Bonaldo *et al*.

(2006) found that soybean meal could be included at levels of up to 30% in the diet of Egyptian sole without any negative effects on weight gain, nutrient retention, performance, feed conversion ratio and specific growth rate. Defatted soybean meal could replace up 40% of the dietary fishmeal protein without significantly affecting growth of juvenile cobia (*Rachycentron canadum*) (Zhou *et al*., 2005). However, feed conversion ratio and protein efficiency ratio in juvenile cobia were negatively affected when the soybean meal replacement of fishmeal exceeded 20% of the diet (Zhou *et al*., 2005). Another study tested soybean meal inclusion in diets for gilthead sea bream (Martinez-Llorens, 2007a), where it was found that juvenile gilthead sea bream were able to tolerate soybean meal inclusion levels up to 30% in the diet without any negative effects on their growth (Martinez-Llorens, 2007a). However, when the juvenile gilthead sea bream grew to about 70 grams, they could tolerate diets with 40 to 50% soybean meal without any significant changes in their growth (Martinez-Llorens, 2007a). Moreover, one study showed that soybean meal could completely replace the fishmeal in the diet for African catfish if there was sufficient dietary amino acid supplementation (Goda *et al*., 2007a).

Tilapia, which are extensively cultured worldwide, can tolerate many different types of foreign ingredients in their diets. For instance, Lin *et al.*, (2004) observed that sub adult hybrid tilapia tolerated complete replacement of fishmeal in their diet with dehulled soybean meal (Lin *et al*., 2004). The results for the tilapia fed the soybean meal and the fishmeal based diets were similar with no significant differences in weight gain, protein efficiency ratio, feed conversion ratio and muscle proximate composition (Lin *et al*., 2004). Goda *et al*. (2007b) found comparable results with Nile tilapia fed diets where the fishmeal was entirely replaced with extruded soybean meal or extruded full-fat soybean meal. The tilapia fed the two soybean meal diets performed on par with the tilapia fed the fishmeal diets in terms of growth and feed utilization (Goda *et al*., 2007b). In contrast, a study using juvenile hybrid tilapia found that the weight gain, protein

efficiency ratio and feed conversion of the fish fed diets with complete dehulled soybean meal substitution were significantly lower than the fish fed diets containing fishmeal (Wu *et al*., 2003).

2.4 Pacific hake

Pacific hake, *Merluccius productus*, are found off the West coast of Canada and the United States of America. It is considered the most abundant groundfish species along the northwest coast (FAO-FD, 2006). The neutral flavor and white color of the flesh in this fish make it favorable as a substitute for other types of fish in the seafood industry, especially fish of similar characteristics like cod and pollock (Wessels and Spark, 1973). Compared to other hake species, Pacific hake muscle tissue also has a high content of polyunsaturated fatty acids, such as eicosapentaenoic acid making the fish an ideal marine resource (Wessels and Spark, 1973; Huynh and Kitts, 2009).

However, Pacific hake are highly undervalued and underutilized because of the poor flesh texture. Wild Pacific hake often have a very soft muscle texture that is almost mushy in consistency making it very low quality. The softness has been attributed to stress in the fish, or high fat diets (St-Hilaire *et al*., 1997), and processing, such as poor storage conditions, prolonged storage, inadequate "stun and bleed" techniques and poor handling during fish rigor mortis (St-Hilaire *et al*., 1997). Lastly, one of the main causes for the soft muscle consistency, post mortem, is the presence of *Kudoa* parasites in the tissue (Kabata and Whitaker, 1981).

2.4.1 Kudoa paniformis

The most prominent cause for the poor flesh texture in Pacific hake is a myxozoan parasite, from the genus *Kudoa* (Kabata and Whitaker, 1981); seventy species have been described in marine fish (Whipps and Kent, 2006). These parasites are generally histozoic and reside in the muscle of the fish (Moran *et al*., 1999). *Kudoa* parasites are unique in that they are very host specific and the majority of them are only be found in one species of fish (Moran *et al*., 1999). For example, the soft flesh occurrence has been documented with *K. cruciformum* in Japanese sea perch (*Lateolabrax japonicus*), *K. histolytica* in Atlantic mackerel (*Scomber scombrus*), *K. musculoliquefaciens* in swordfish (*Xiphias gladius*), *K. peruvianus* in Chilean hake (*Merluccius gayi*), *K. clupeidae* in Atlantic herring (*Clupea harengus*), *K. funduli* in mummichog (*Fundulus heteroclitus*) and *K. mirabilis* in ribbonfish (*Trichiurus haumela*) (Moran *et al*., 1999).

Kudoa paniformis, the parasite that specifically targets Pacific hake, causes myoliquefaction of the fish skeletal muscle from proteolytic processes, post mortem (Adlerstein and Dorn, 1998). The parasite is mainly found off the coast of British Columbia in the northeastern region of the Pacific Ocean (Adlerstein and Dorn, 1998). It has been reported to have infected over 50% of the wild Pacific hake population (Kabata and Whitaker, 1986). Approximately 20% of Pacific hake that are two years and older have already been infected (Adlerstein, 1992), with the percentage of infection increasing with age and size of the fish (Kabata and Whitaker, 1986). There have been reports of several hake with over 80% of their muscle fibers infected with the parasite (Adlerstein and Dorn, 1998). These parasites are quadrate shaped, with rounded valve tips and pyriform polar capsules (Kabata and Whitaker, 1981). Infected muscle tissue contains high amounts of cathepsin L-like proteases that break down connective tissue and muscle proteins during postmortem storage and processing (Samaranayaka *et al*., 2006). Not only does the

parasite affect muscle texture, studies have shown that it can also significantly reduce the fecundity of Pacific hake, with higher levels of infection causing greater drops in fecundity (Adlerstein and Dorn, 1998).

The entire life cycle of the *Kudoa* parasite is unknown, but some specific parts of the sporogenic process within the fish have been documented. Infection usually occurs through the gills of the fish with water as a medium, although absorption of spores through the gastrointestinal tract is also possible (Moran *et al*., 1999). The early generative parasite cells will attach to the muscle cells and eventually mature into spores (Stehr, 1986). The number of spores will gradually increase from the infected muscle and expand into the muscle fibers (Kabata and Whitaker, 1981). At this stage, the parasite will produce proteolytic enzymes that break down the muscle cells, which in turn, replaces the muscle cells with the parasite, which then has space for growth and development (Moran *et al*., 1999). Muscle fibers will initially contain white pseudocysts, which are fibers containing viable spores because of the lack of an immunological host response (Adlerstein and Dorn, 1998). An immunological host response is finally triggered when the number of spores increases to the point where the spores contact the inner surface of the sarcolemma fiber (Kabata and Whitaker, 1981). The immune system will try to encapsulate the pseudocyst of spores to degrade and destroy the parasite, resulting in a smaller black pseudocyst (Whitaker and Kabata, 1987). The black pseudocysts consist of destroyed spores due to host immune reactions and are inactive parasitic spores (Adlerstein and Dorn, 1998). Inflammation around the pseudocysts is the primary method used by the host to combat the infection (Lom and Dykova, 1992). Therefore, it is quite possible for the host to eventually fight the parasite infection. However, once the host is deceased, proteolytic enzymes from the spores will degrade the muscle tissue leading to the soft postmortem fillet texture (Moran *et al*., 1999).

2.4.2 Kudoa thyrsites

Although most *Kudoa* parasites are specific to only one host, there are a few exceptions. Pacific hake can potentially exhibit co-infections of both *Kudoa paniformis* and *Kudoa thyrsites* spores (Moran *et al*., 1999). Tsuyuki *et al*. (1982) reported that *Kudoa thrysites* infections in Pacific hake did not lead to a soft muscle texture and the degradation of muscle tissue was caused by proteolytic activity associated with the *Kudoa paniformis*. A more recent study determined that Pacific hake muscle deterioration could also be caused by *Kudoa thyrsites* infections, which at low infection rates may have an even greater impact than *Kudoa paniformis* on Pacific hake muscle quality (Zhou and Li-Chan, 2008). In contrast to other *Kudoa* species, *Kudoa thyrsites* is a unique parasite in that it occurs worldwide and displays a wide range of host specificity (Dawson-Coates *et al*., 2003; Funk *et al*., 2007). *Kudoa thyrsites* has been documented in over twenty different fish species (Moran *et al*., 1999). One possible explanation for this widespread infection rate is that aquaculture growth has led to the culturing of new fish species in new geographical areas, like the Atlantic salmon in the Pacific Ocean. In addition, native fish species are cultured in different environmental conditions, such as Coho salmon in marine net pens. This would help introduce *Kudoa thyrsites* to a new area that was once free of the parasite (Kent, 2000).

Kudoa thyrsites has a significant detrimental effect on the salmon aquaculture industry because of the hosts it targets, such as Atlantic and Coho salmon (Whitaker and Kent, 1991). Chinook salmon are much more resistant to *Kudoa thyrsites* infections than other farmed salmon species (Whipps and Kent, 2006). In North America, especially in British Columbia, salmon infected by *Kudoa* is a serious economic problem because the majority of farmed salmon are either Atlantic or Coho (Dawson-Coates *et al*., 2003). In British Columbia, approximately 76% of the farmed salmon production is Atlantic salmon, with Coho salmon being the third most widely produced

at approximately 2% (Kent, 2000). Since *Kudoa thrysites* is worldwide, Atlantic salmon infections have been reported in Spain and Ireland as well (Kent, 2000). Interestingly, very few of the immature salmon population are infected, but the prevalence of the infection increases as the salmon sexually mature (Moran *et al*., 1999). Similar to the problems associated with *Kudoa paniformis* in Pacific hake, heavy infections of *Kudoa thrysites* results in white and black cysts in the muscle fibers (Dawson-Coates *et al*., 2003) and myoliquefaction in the host post mortem (Moran *et al*., 1999; St-Hilaire, et al., 1997). The parasite causes no apparent health risks to the living fish (Dawson-Coates *et al*., 2003) or host mortality (Whipps and Kent, 2006). Therefore, there are no signs of disease in the fish, making an infection problematic to determine by observing the physical appearance and behavior of the fish alone. The muscle tissue degrades to a soft consistency only after death of the infected fish. Since aquaculture depends on high quality products, unattractive cysts and soft muscle texture in fish can have a major negative economic impact on the industry (Moran *et al*., 1999).

Thus, due to the co-infection found in Pacific hake, extreme caution must be taken when preparing Pacific hake products that are fed to other fish. Ideally, the fish products should be subjected to filtering methods in order to remove the *Kudoa* parasites. Since the parasites can range in size from 5 to 10 μm, a filter system can be easily incorporated to remove the parasites (Moran *et al*., 1999).

2.5 Fish protein processing

Approximately 30% of the fish processed globally is used to create fishmeal and 50% of this fishmeal is considered fish waste and byproducts (Kristinsson and Rasco, 2000). These fish processing byproducts are generally discarded, but the levels of unwanted fish material can accumulate to very high levels and the proper treatment and refining of such waste can become

very costly and ecologically harmful. Also, there have been several regulations restricting industrial processors to continue to dump such large quantities of fish waste into the ocean (Kristinsson and Rasco, 2000). Protein hydrolysis processes have been developed to try to resolve these environmental issues. That technology would allow the industry to recover and modify the proteins from the fish waste to produce a broad array of industrial products, functional foods, and food ingredients (Kristinsson and Rasco, 2000). Currently, there are few hydrolyzed fish protein food ingredients, such as East Asian sauces and condiments. Hydrolysates can display high solubility, high whipping ability and low viscosity making them an asset in certain food products (Adler-Nissen, 1979). The marine by-products are very rich in protein and can be processed, through hydrolysis, to recover the valuable and potentially nutritious protein, peptide and amino acid hydrolysates (Kristinsson and Rasco, 2000; Aksnes *et al*., 2006c).

2.5.1 Fish protein hydrolysates

In the past few years, fish protein hydrolysates have been the focus of many studies. Hydrolysates are proteins that are denatured and broken down into smaller proteins, peptides, and free amino acids, through the application of chemical or biological processes (Kristinsson and Rasco, 2000). The breaking down of proteins into hydrolysates can be accomplished with acids, bases, endogenous enzymes, and digestive and bacterial proteases (Kristinsson and Rasco, 2000). In chemical hydrolysis, acids and bases are used to cleave peptide bonds to break down the proteins. However, this technique has its limitations because it is very difficult to control and reproduce, which leads to inconsistent end products that have different compositions and functions (Kristinsson and Rasco, 2000). Acids and bases also require high temperatures and extreme pH environments, which would generally lower the nutritive value and functional properties of the hydrolysates (Kristinsson and Rasco, 2000). Endogenous enzyme-based

biological methods have the advantage of being inexpensive, since they rely on the use of the naturally occurring digestive enzymes found within the fish body to digest the proteins during the hydrolysis. These digestive enzymes include trypsin, chymotrypsin, and pepsin proteases within the gastrointestinal tract, the enzymes within the fish viscera and catheptic and lysosomal proteases within the fish muscle (Kristinsson and Rasco, 2000). Exogenous enzyme-based biological methods, employed to produce hydrolysates, are used much more frequently because the process is easier to control and replicate than the two aforementioned methods (Kristinsson and Rasco, 2000). Also, when the hydrolysis is carried out in a firmly controlled environment, the end product tends to exhibit desirable functional properties and it retains all of the nutritional functions due to the use of less extreme hydrolysis conditions (Adler-Nissen, 1979). External enzymes are added to help cleave the peptide bonds and the degree of hydrolysis is controlled by the specific enzyme as well as the hydrolysis environment, in terms of pH, time and temperature (Kristinsson and Rasco, 2000).

The production of fish protein hydrolysates has the potential to use both fish byproduct wastes and underutilized fish species, such as Pacific hake, that are of low quality and would otherwise be discarded (He *et al*., 2006). Whole fish can be used to produce fish protein hydrolysates because other components of the fish, such as viscera and bones are also important sources of nutrients (Aksnes *et al*., 2006a). Studies have been conducted to demonstrate that hydrolysates can be made into nutritional supplements and functional proteins for human consumption (He *et al*., 2006). There is a lot of promise for the protein hydrolysis technology, but it does have several limitations. The commonly used enzymatic hydrolysis process can be quite costly and fish processing industries would rather discard the wastes if the hydrolysis costs surpass those of waste treatment (Kristinsson and Rasco, 2000). Another limitation is the creation of bitter tasting peptides, which are organoleptically unfavorable for consumption by humans and possibly for

consumption by fish. These limitations have encouraged researchers to look at applications of hydrolysates that utilize specific functional properties at lower levels, for example as dietary feed attractants, immunity enhancers and growth promoters for farmed fish (Espe *et* al., 1992). Fish protein hydrolysates could also be an ideal ingredient for aquaculture feeds because unlike the various plant proteins used in fish feeds, fish proteins contain a well balanced profile of essential amino acids.

Many studies have already been conducted to test the potential of fish protein hydrolysates or fish silage as a feed ingredient in aquaculture. Fish silage is a higher moisture compound than fish protein hydrolysates and the former is produced by enzymatic break down of the proteins with the aid of an acidic environment (Espe *et* al., 1992). Peptide fractions from acidic hydrolysis act as adjuvants for certain vaccines and stimulants for the fish immune system (Gildberg *et al*., 1996). Other studies have looked into using fish protein hydrolysates as a feed ingredient to help combat certain infections, like *Aeromonas salmonicida* in Atlantic salmon (Gildberg *et al*., 1996). Different fish protein hydrolysates and fishmeals produced from different raw materials and processes have been compared to help determine the ideal combination of dietary fish ingredients (Anderson *et al*., 1992; Anderson *et al*., 1995). Hydrolyzed fish protein in the form of fish silage was used as a replacement for a portion of the fishmeal in Atlantic salmon diets (Sveier *et al*., 2001a). The results have shown that when included in the diet fish silage can significantly increase specific growth rate (Sveier *et al*., 2001a). Aksnes *et al*. (2006b) reported that fish protein hydrolysates and plant proteins differ in more than just the amino acid profile, mineral content, and macronutrients. There are also specific smaller molecular weight compounds in fish protein sources that make them superior to plant based proteins when used as a dietary supplement for fish. Some of these compounds include nucleotides, taurine and histidine peptides such as carnosine and anserine (Aksnes *et al*., 2006b). These compounds can

act as growth promoters or feed attractants, which would explain why certain fish in aquaculture could not efficiently adapt to a completely plant-based diet because of the absence of these compounds. Fish protein hydrolysates were evaluated for their role as feed attractants to help enhance the palatability of fish diets. A study with Atlantic cod showed that removing the smaller molecular weight fraction from fish hydrolysates significantly reduced the growth of the fish (Aksnes *et al*., 2006b). Therefore, small compounds in this fraction are essential for optimal growth and feed utilization for Atlantic cod. This finding coincides with another study, which showed the most crucial aspect of shrimp protein hydrolysates that would indicate a high quality product is their degree of hydrolysis because of the presence of small molecular weight compounds that are produced (He *et al*., 2006). Therefore, further studies should look into the production of different types of fish protein hydrolysates that have different peptide and free amino acid profiles. Once their compositions have been identified, these hydrolysates could then be tested as feed attractants.

2.6 Feed attractants

Feed attractants are defined as any type of compound that could be added as a feed ingredient to a diet to help enhance the overall palatability of the diet. Therefore, feed attractants for fish are generally compounds derived from or are of similar chemical composition to naturally occurring compounds that the fish are familiar with and are attracted to. For example, krill is an important natural prey for many carnivorous fish and thus, krill based ingredients would act as natural feed attractants and taste enhancers when applied to the diet (Suontama, 2007a). Feed attractants are usually in the form of small chain peptides, small molecular weight compounds and free amino acids, which are what fish taste buds sense to detect taste (Sutterlin and Sutterlin, 1970).

Feed attractants are applied either internally or externally onto the formulated feed. Internal applications involve mixing the attractant into the homogenous mash before pelleting and the attractants encourage fish to continue eating after feeding has already begun (Kasumyan and Doving, 2003). In contrast, external applications involve mixing the attractant into the oil fraction of the formulated diet and applying the oil-attractant mixture as a top coating. This approach draws the animal towards the feed and helps to initiate feed consumption (Kasumyan and Doving, 2003). Feed attractants can work in three ways. Firstly, they can add recognizable and familiar flavors to the feed that were not there initially, such as adding krill meal to a diet. Secondly, attractants can replace lost flavors that have been taken out of the diet, such as replacing some of the marine flavors lost by removing the fishmeal portion of the diet. Thirdly, feed attractants can be used to overpower and mask the feeding deterrents that may be present in the diet. This is generally the case when alternative plant ingredients are used and feed attractants are required to help cover up the unpalatable tastes found within the plant ingredients.

Among all animals, fish are one of the most sensitive to tastes and therefore, to changes in their diets (Kasumyan and Doving, 2003). Although variable between fish species, a vast number of taste buds are located in their oral cavity, esophagus, pharynx, lips, gills, fins, barbells and over the surface of their entire body (Kasumyan and Doving, 2003). Different sensory compounds can induce different eating behaviors in fish. Suppressant substances detected by the extraoral taste system, are known to lower the degree of grasping in fish (Kasumyan and Doving, 2003). In contrast, incitants are also signaled by the extraoral gustatory system. However, these increase the level of grasping, snapping, suction, biting, tearing and any other actions that mimic the capture of food by fish (Kasumyan and Doving, 2003).

The two types of compounds that trigger the oral gustatory system are stimulants and deterrents (Kasumyan and Doving, 2003). In terms of palatability, these two are the most important factors because they determine the final acceptance of the food by fish. Stimulants are any materials that increase the swallowing and ingestion of food; at the opposite end, deterrents promote the rejection and abandonment of food (Kasumyan and Doving, 2003). The fish immediately discharge food that contains deterrents after the food has initially been grasped and the fish will refrain from recapturing the food (Kasumyan and Doving, 2003).

Other types of compounds, known as enhancers, are also detected by the oral gustatory system (Kasumyan and Doving, 2003). Enhancers are similar to stimulants, but instead of adding new flavors to the diet, they enhance the already existing stimulating flavors found within the food (Kasumyan and Doving, 2003).

The types of feeding behavior induced in fish can also be used to classify feed attractants. There are generally five different behavior patterns that fish display in response to certain feed ingredients (Mearns, 1986). The most common is swim behavior, where the fish moves forwards, upwards, or backwards. Movement or a jerk of the fish body without a change in position is another common behavior. These two behaviors are associated with the search for food in the wild (Mearns, 1986). Snapping, the sudden opening and closing of the mouth, and darting, short bursts of speed, are both associated with the chase and capture of prey in the wild (Mearns, 1986). The fifth behavior, which is not very common, is yawning or the slow opening and closing of the mouth (Mearns, 1986).

The types of compounds that fish find stimulating and palatable are dependent entirely on the fish age and species. Certain substances seem to have similar effects in a large number of fish

species. Physiologically, fish have taste receptors that are very sensitive to free amino acids, which create electrophysiological responses (Kasumyan and Doving, 2003). In many fish species, natural L-isomers of amino acids are more accepted as feeding stimulants than their D-enantiomer counterparts (Kasumyan and Doving, 2003). Neutral amino acids with unbranched side chains and a low number of carbon atoms are commonly stimulatory in fish, whereas acidic amino acids elicit low gustatory stimulations, while responses to basic amino acids are dependent on the fish species (Kasumyan and Doving, 2003). Although single amino acids can be quite stimulatory, certain amino acids enhance the electrophysiological response of other amino acids making amino acid combinations more effective for certain fish (Marui and Kiyohara, 1987). Peptides can act as stimulants in fish, but they are far less effective than their component free amino acids (Caprio, 1978).

Many types of small molecular weight compounds are being investigated as potential feed attractants for various fish species used in aquaculture. These compounds include organic acids, sugars, aldehydes, alcohols, amines, hydrocarbons, nucleotides, nucleosides, betaine and free amino acids (Kasumyan and Doving, 2003).

2.6.1 Feed attractants for salmon

Extensive research has been conducted on the taste preferences of Atlantic salmon because of their popularity in the aquaculture industry. One study attempted to use 5% fish protein, squid, and stick water hydrolysates as feed attractants in diets without fishmeal for Atlantic salmon (Espe *et al*., 2006). The fish fed the diets devoid of fishmeal showed signs of reduced feed intake and lower growth compared to the fish fed a control diet with fishmeal (Espe *et al*., 2006). Another study also looked at fish protein hydrolysates as a feed attractant, using the hydrolysates at different concentrations (Refstie *et al*., 2004). Inclusion of fish protein hydrolysate at 10 or

15% in place of the fishmeal in the diet increased feed consumption by Atlantic salmon (Refstie *et al*., 2004). Fish protein hydrolysate can act as an effective feeding stimulant and a protein feed ingredient because of its high digestibility value (Refstie *et al*., 2004). Fish protein hydrolysate made from a "CPSP Special G" mixture from France, when used in place of 5% of the amino acid nitrogen, was also effective in increasing growth rate of Atlantic salmon fry (Berge and Storebakken, 1996). Fish silage made from saithe was also tested as a feed attractant when included at 20% of the dietary protein. This silage containing diet promoted increased weight gain and feed utilization of Atlantic salmon (Espe *et al*., 1992).

A very popular feed ingredient used in the aquaculture industry for salmonids is crustacean meal, especially krill meal. These crustaceans are currently an abundant, but underused, marine product (Olsen *et al*., 2006). Suontama *et al*. (2007a) experimented with various types of crustacean meals, including Antarctic krill (*Euphausia superba*), northern krill (*Thysanoessa inermis*), and Arctic amphipods (*Themsto libellula*), as replacements for up to 60% of the fishmeal protein in Atlantic salmon diets. In comparison with fish fed the fishmeal control diet, feed conversion ratio, fish muscle composition, and nutrient digestibility were all similar for the fish fed diets with crustacean meal as a feed ingredient (Suontama, 2007a). The inclusion of krill in the diets did not increase the specific growth rate of the Atlantic salmon (Suontama *et al*., 2007b). In comparison, another study found that Atlantic salmon fed diets containing 20 to 60% Antarctic krill meal as the dietary protein, exhibited better growth compared to those fed the fishmeal control diet (Olsen *et al*., 2006). However, these results were not observed in the latter half of the experiment, after the first 71 days of feeding, where there were no growth difference among the Atlantic salmon fed the different diets (Olsen *et al*., 2006). The authors attributed these results to the fact that krill meal is, firstly, a feed attractant and feed attractants tend to lose their effectiveness over time (Olsen *et al*., 2006). The similar amino acid compositions do

however indicate that krill meal can act as an effective replacement for fishmeal in Atlantic salmon as either a feed attractant or a protein replacement (Olsen *et al*., 2006). Similar results regarding weight gain were reported when krill meal was used as a supplement in diets for juvenile Chinook salmon (Anderson *et al*., 1997).

An extensive study, conducted in 1970 by Sutterlin and Sutterlin, looked at various compounds that could potentially be stimulatory for Atlantic salmon. These substances were not tested with live feeding, but by responses measured from the facial and palatine nerves of the fish (Sutterlin and Sutterlin, 1970). Sixteen different amino acids, including L-proline, glycine, L-isoleucine, L-leucine, L-methionine, and L-taurine, each at a concentration of 0.01 M failed to elicit a response in the facial nerves of the Atlantic salmon (Sutterlin and Sutterlin, 1970). When the sixteen amino acids were tested at the same concentration on the palatine nerves, only proline gave a consistent positive response (Sutterlin and Sutterlin, 1970). Other compounds that brought forth a response in the Atlantic salmon nerves were chloride salts, organic acids, and minerals (Sutterlin and Sutterlin, 1970). In contrast, another study found that feed intake and growth of Atlantic salmon significantly increased when crystalline methionine was included at 0.5% of the diet that contained soy protein concentrate (Sveier *et al*., 2001b). However, the salmon growth performance was still inferior to the growth of salmon fed the control diet without any soy protein concentrate (Sveier *et al*., 2001b). The preceding methionine results were strengthened by the findings from another experiment where 5 μM L-methionine was effective in creating a positive response in Atlantic salmon fry (Mearns, 1986). The study also showed that 5 μM L-proline and L-alanine were very strong feeding attractants for Atlantic salmon (Mearns, 1986). Hara *et al*. (1994) also found that L-proline, along with L-α-amino-β-guanidinopropionic acid (Ll-AGPA), were strong stimulants for Atlantic salmon.

2.6.2 Feed attractants for trout and charr

Many different types of trout species have been studied because of their high use in aquaculture and their similarities with salmon species. Trout and salmon species have a very limited response spectrum for detecting taste senses, but are highly sensitive to a few types of stimulants (Hara and Zielinski, 1989; Kohbara and Caprio, 2001), unlike other fish like catfish (Hara *et al*., 1993). Like the salmon species, L-proline has been found in several studies to be a strong feeding stimulant for brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) (Hara *et al*., 1994; Mearns, 1986). These two salmonid species also displayed positive responses to L-alanine (Mearns, 1986; Sutterlin and Sutterlin, 1970).

Many studies have revealed that the L-amino acids are potential feed attractants, whereas the D-amino acids act as deterrents because the gustatory system of rainbow trout is stereospecific for only the L-forms of amino acids (Adron and Mackie, 1978). It has also been determined that salmonid species contain four main types of receptor sites for receiving stimulations, i.e., Pro (proline, hydroxproline, and alanine), AGP (α-amino-β-guanidinopropionic acid, and betaine), Phe (phenylalanine and leucine), and Arg (arginine) receptors (Hara *et al*., 1999). This discovery helps to explain the results from many rainbow trout experiments with respect to their sensitivity to different types of feed attractants. For instance, Hara *et al*. (1999) found that proline, phenylalanine, arginine, and α-amino-β-guanidinopropionic acid had stimulatory effects on the palatine nerves of rainbow trout at a concentration of 10^{-3} M. Proline and arginine also produced positive responses in rainbow trout when applied at concentrations of 10^{-3} to 10^{-4} M to a cotton soaked pellet (Jones, 1989). Moreover, Marui *et al*. (1983) found that the use of betaine in rainbow trout diets was very effective in increasing feed intake. However, Jones (1989) discovered that although betaine and alanine are effective feed attractants, they did not produce a response on the fish gustatory receptors at concentrations of 10^{-3} to 10^{-4} M. This indicates that

nerve stimulation and response are not the only factors that determine the palatability of a substance. Yamashita *et al*. (2006) also found similar results with proline, leucine, alanine, and betaine which produced highly positive responses in rainbow trout at a concentration of 10^{-3} M. Again, proline was the most potent amino acid for stimulating the fish at concentrations as low as 10^{-8} M. L-proline, L-leucine, and α-amino-β-guanidinopropionic acid, at a concentration range of 10^{-2} to 10^{-3} M, produced strong stimulatory effects in the facial and glossopharyngeal nerves of the rainbow trout (Kohbara and Caprio, 2001).

All of these studies have only looked at testing specific small molecular weight compounds for their efficacy as feed attractants or their ability to alter sensory nerve responses in rainbow trout. By contrast, Davies and Morris (1997) investigated how certain amino acids helped increase the palatability of the diet for rainbow trout by replacement of 66% of the herring fishmeal in the diet with solvent extracted soybean meal. Various combinations of amino acids, e.g., methionine, threonine, lysine, tryptophan, histidine, and arginine were evaluated in the soybean meal diets at a range of 0.25 to 0.5% of the diet (Davies and Morris, 1997). There were no significant increases in growth, energy utilization or feed efficiency in the rainbow trout fed the soy diets compared to the rainbow trout fed the fishmeal control diet (Davies and Morris, 1997). However, the soy diet supplemented with all six amino acids produced significantly better trout growth rate and feed efficiency when compared with the responses of trout fed the soy diet without any amino acid supplementation (Davies and Morris, 1997).

Unlike the other studies done on salmon, a study by Adron and Mackie (1978) found that glycine and proline were ineffective feeding stimulants for rainbow trout, whereas proline was a feed repellent (Adron and Mackie, 1978). Single amino acids were not very effective in stimulating the taste senses, but combinations of amino acids, like tyrosine, phenylalanine, and lysine each

added at 0.01% of the diet, worked very well. The authors hypothesized that the levels of these amino acids better mimicked those found in prey ingredients (Adron and Mackie, 1978). Other studies have looked into the use of taurine, anserine, nucleic acids, and nucleotides for enhancing trout growth, feed intake, and their overall health (Aksnes *et al*., 2006c). Other types of compounds that have proven to be effective as dietary feed attractants for rainbow trout include krill hydrolysate at a 2% replacement of the fishmeal and thin corn distillers solubles (Oikawa and March, 1997; Thiessen *et al*., 2003).

Jones (1990) also tried to test some compounds as feed stimulants and attractants in rainbow trout that were not evaluated in other studies. These chemicals included aldehydes, amides, amines, sugars, alcohols, and untested amino acids (Jones, 1990). Although none of the chemicals tested were as effective as some of the amino acids like proline and alanine, tested in other studies, there were still some, such as octanol, hexanol, L-norvaline, D-ribose, D-glucose, and sucrose that produced a palatability effect at high concentrations of 1 to 0.1 M (Jones, 1990).

2.6.3 Feed attractants for other fish

Many other types of fish species have been used in feeding trials and nerve response experiments to test the efficacy of certain chemicals as dietary feed attractants and stimulants. Although many of these species are different physiologically from salmonid species, like salmon and trout, there are a few similarities about the types of chemical feeding stimulants and attractants identified. Feed intake was noted to be improved in *Tilapia zillii* when their diets included aspartic acid, glutamic acid, alanine, serine, or lysine (Adams *et al*., 1988; Johnsen and Adams, 1986). Similar to the salmonid species and tilapia, channel catfish responded well to L-alanine and L-arginine, as well as L-serine (Caprio, 1975). A feed stimulating mixture containing 0.4% alanine and 0.6% serine was also effective in increasing feed intake in Dover sole (Papatryphon and Soares, 2001).

Proline, the most potent amino acid feed attractant for salmonids, was effective at 10^{-3} M in stimulating the olfactory and gustatory nerves of Atlantic halibut, *Hippoglossus hippoglossus* (Yacoob and Browman, 2007). Proline receptors are present in almost all fish species that have been studied (Marui and Caprio, 1992). Inosine-5-monophophate was another compound found to be an effective feed attractant at concentrations of 10^{-3} M for large mouth bass and Atlantic halibut (Oliveira and Cyrino, 2004; Yacoob and Browman, 2007).

Other compounds that have been tested and seen to be effective as dietary feed attractants and stimulants are krill hydrolysate for yellow perch, lake whitefish and walleye, and fish protein hydrolysates, made from Atlantic salmon heads and backbones, for Atlantic cod (Kolkovski *et al*., 2000; Aksnes *et al*., 2006b). The use of an attractant mixture comprised of 0.4% alanine, 0.6% serine, 0.2% inosine-5'-monophosphate, and 0.4% betaine was effective in striped bass (Papatryphon and Soares, 2000).

Betaine is a compound that has been extremely effective in many studies on many species of fish. For example, Reig *et al*. (2003) found that betaine encouraged Dover sole to continue feeding when it was applied internally to the feed. Similar results were seen with the use of mussel (*Mytilus edulis)* flesh or extract, which contains a high concentration of betaine (1.62%) and taurine (0.76%), on Dover sole (Mackie *et al*., 1980), and using mussel extract in the diet increased appetite of gilthead sea bream (Tandler *et al*., 1982). Papatryphon and Soares (2001) found similar results with striped bass using a feed stimulant mixture containing 0.4% betaine. Betaine is an effective stimulant for Atlantic halibut as well, but only at a high concentration of 10^{-2} M, which indicates that it is released when prey is bitten or injured (Yacoob and Browman, 2007).

2.6.4 Feed Attractants for Crustaceans

Crustaceans also play an important role in the aquaculture industry. Inexpensive plant and terrestrial proteins, which have also been used in this type of aquaculture, have led to poor growth performance (Nunes *et al*., 2006). Many studies have looked into using feed attractants to help make the current crustacean diets or the less expensive plant protein based diets more palatable for crustaceans. Smith *et al*. (2005) found that black tiger shrimp, *Penaeus monodon*, had a high preference for crustacean and krill meal. Similar results were also seen in another study, where crustacean and krill meal were effective feed attractants for black tiger shrimp (Huang *et al*., 2003). Black tiger shrimp also recognized betaine, nucleotides, glycine, arginine, alanine, taurine, and glutamic acid as effective attractants when added to the feed at 0.5 to 5% of the diet, especially when used in combination (Smith *et al*., 2005). Similarly, the aforementioned amino acids, along with glutamine, isoleucine, and serine also ellicited positive feeding behaviors in another study with black tiger shrimp at concentrations ranging from 10^{-2} to 10^{-6} M, while nucleotides and adenosine 5'-monophosphates produced very little response in the shrimp (Coman *et al*., 1996). Overall, betaine and taurine were very effective feed attractant compounds for black tiger shrimp (Coman *et al*., 1996). This is probably because, in nature, both betaine and taurine are released from damaged tissues found in prey of black tiger shrimp (Coman *et al*., 1996). Thus, the detection of these compounds would indicate injured or freshly killed prey (Coman *et al*., 1996). Taurine is also steadily released from various marine invertebrates, which could indicate living prey for the black tiger shrimp (Coman *et al*., 1996). In contrast, betaine as a feed ingredient included at 3% wet basis was not an effective feed ingredient for Pacific white shrimp, *Litopenaeus vannamei* (Nunes *et al*., 2006). However, whole squid protein hydrolysates, enzymatically digested bivalve mollusks, and fish soluble proteins provided stimulatory responses in Pacific white shrimp when added at a 3% wet basis level to the feed (Nunes *et al*., 2006). Squid products, in the form of squid muscle extract, and fish proteins, in the form of cod

extracts, were also highly effective as feed attractants for lobster, *Homarus gammarus* (Mackie, 1973). These extracts are rich in glycine (9.8%), proline (16.1%), and taurine (3.7%), which have repeatedly been shown in many studies to be strong feed attractants (Mackie, 1973).

3.0 THESIS OBJECTIVES AND HYPOTHESES

The intent of this study was to determine whether Pacific hake, undervalued due to their poor postmortem fillet texture, could be used to produce a fish protein hydrolysate that, in turn, would have appetite-enhancing properties when included in the diet for juvenile Chinook salmon. The study was conducted on juvenile fish due to their higher taste sensitivity. Chinook salmon were chosen because of their dislike for the taste of soybean protein products (Bureau *et al*., 1998). Therefore, the present study set out to test the effectiveness of the feed attractants to mask the taste of soybean meal in the feed, an ingredient Chinook salmon find aversive.

The first goal of the research was to chemically characterize various test fish protein hydrolysates and as a second goal, select hydrolysates for use as potential feed attractants for the palatability feeding trial on juvenile Chinook salmon. Chemical characterization involved assessing the degree of hydrolysis of the different hake protein hydrolysates and it considered their respective peptide and free amino acid profiles. In the second research objective, hake protein hydrolysates with two different degrees of hydrolysis were studied for their potential to act as feed attractants for juvenile Chinook salmon when each of these was applied as a component in the oil top dressing of the feed containing soybean meal. The degree of diet palatability was assessed by conducting feeding trials on pre-smolt Chinook salmon in which several palatability parameters were measured for fish on the soybean meal based diets that were coated with each of the different fish protein hydrolysates or commercial feed attractants. The experiment also followed the palatability responses of groups of fish fed a negative control soybean meal based diet without any feed attractant and a positive control diet without soybean meal. The third objective of this research was to assess the potential risk posed by using hydrolysates prepared from *Kudoa* parasitized Pacific hake as feed attractants, by quantifying the

Kudoa spore counts in the Pacific hake starting material, hydrolysates, and subsequently in the Chinook salmon themselves that had been fed diets incorporating hydrolysates as feed attractants.

The major null hypotheses tested in this thesis were as follows:

(1) Ho_1: Pacific hake fish protein hydrolysate, as a top coating feed constituent, will not increase the palatability of fish feed containing soybean meal for cultured juvenile Chinook salmon.

(2) Ho_2: The EWOS commercial feed attractants, as a top coating feed constituent, will not increase the palatability of fish feed containing soybean meal for cultured juvenile Chinook salmon.

(3) Ho_3: Pacific hake fish protein hydrolysates will not be a risk for contaminating juvenile Chinook salmon with *Kudoa* spores, when used in the oil top coating of their diets.

4.0 MATERIALS AND METHODS

4.1. Feed attractants and feed ingredients

4.1.1. Pacific Hake

The Pacific hake were provided by Nicholas Knott (Steveston Seafood Direct Ltd, Vancouver, British Columbia) and Ron de Silva (Pacific Fisheries Technologies, Inc. Vancouver, British Columbia). Forty-one hake with a mean weight and length of 1033.3 g (SE, 106.4 g) and 53.0 cm (SE, 1.5 cm) respectively were used in this study. The fish were representative of the average size and spore infection for wild, summer Pacific hake. They were caught on August 13th and 15th, 2005 in the waters off Vancouver Island (48° 32' 03.0"N to 48° 30' 57.9"N and 124° 49' 56.5"W to 124° 43' 39.0"W). The fish were from eight different batches, with four batches being caught on each day at approximately 7:00 am, 9:00 am, 11:00 am, and 1:00 pm. Fish were frozen on the fishing vessel immediately after being caught and transported on ice to our laboratory 1 to 3 days later. Upon arrival, they were stored in a -25°C freezer until their use for production of fish protein hydrolysates, at which time they were thawed overnight in a 4°C cold room.

4.1.2. Fish protein hydrolysate production

Four different Pacific hake fish protein hydrolysates, labeled ALC1 (1 hour Alcalase), ALC4 (4 hour Alcalase), FLA1 (1 hour Flavorzyme), and FLA4 (4 hour Flavorzyme), were prepared according to the procedures described by Khan and Li-Chan (2008), using two Novozyme® commercial proteolytic enzyme preparations (donated by Brenntag Canada Inc, Langley BC) and two hydrolysis times (Table 4.1).

Thawed, whole Pacific hake (41 in total), cut into large pieces were ground twice through a grinder (BEEM Gigant, Butcher & Packer Supply Company, Michigan) with a 4 mm screen. The

fish were then ground to a coarse slurry mixture and mixed together (Khan and Li-Chan, 2008). Seven kg of the fish slurry mixture were used for each hydrolysis treatment. The slurry was first mixed with 14 L of distilled, deionized water and then heated to 50ºC in a 25 L stainless steel pot on a gas burner. Twenty-six mL of Flavorzyme 500 L (500 LAPU/g) or Alcalase 2.4 L (2.4 AU/g), were then added to the fish slurry mixture to yield 3% (w/w) enzyme:protein substrate ratio. The slurry was hydrolyzed for 1 or 4 hours at 50ºC with constant stirring. The insoluble portion and bone fraction of the slurry were removed by filtering through a cheesecloth. Subsequently, the hydrolyzed samples were immediately placed in a cold room (4ºC) for 24 hours to be cooled down.

All samples were centrifuged at 2800Xg for 15 minutes and filtered with a double-layered cloth fiber net (Khan and Li-Chan, 2008). Then each sample was heated to 80ºC using a gas burner and maintained at 80ºC for 15 minutes, in order to pasteurize the sample and inactivate *Kudoa* parasites. The heated sample was placed in a 4ºC cold room and cooled to approximately 20ºC. At this stage the fat, which became visible as cloudy white fractions in the liquid, was removed by pipette. The liquid samples were poured into aluminum trays, covered with aluminum foil, and placed in a -30ºC freezer. The frozen samples were freeze dried, at a condenser temperature of -50ºC and a vacuum of 28" Hg, to create a very fine and dry particulate powder, which was placed in 50 mL airtight, plastic test tubes that were flushed with nitrogen to minimize lipid oxidation during storage. The test tubes placed in airtight Ziploc bags with a desiccant were stored in a -30ºC freezer until further use.

4.1.3 Fish protein hydrolysate recovery

Fish protein hydrolysate recovery was assessed by measuring the final yield compared with the initial weight of the starting material. The final weight of each freeze dried sample was measured

and divided by the starting weight to give the fish protein hydrolysate recovery values as wet weight based values. Dry weight based values were calculated based on the dry material content of the whole fish mince, which was predetermined by the vacuum oven drying method (AOAC Official Method 934.01).

4.1.4 Commercial feed attractants and raw materials

The commercial attractants included a South American Antarctic krill meal (*Euphausia superba*) from Hinrichsen Trading Company (Santiago, Chile). Two experimental commercial feed attractants, labeled as "screen 1" and "screen 2", as well as other ingredients used in the pelleted feed including standard commercial anchovy meal, corn gluten meal, and soybean meal were obtained from EWOS Canada (Surrey, British Columbia).

4.1.5. Proximate composition analysis

The protein contents of the four Pacific hake fish hydrolysates were measured using a Leco nitrogen analyzer for total nitrogen content (Technicon industrial method No. 334-74W/B, revised March, 1977, Technicon Industrial Systems, Tarrytown, NY, USA). The percent nitrogen was multiplied by 6.25 to obtain the percent crude protein content (Higgs *et al*., 2006). Samples were dried for 1 hour at 100°C prior to determination of lipid content using a Goldfish apparatus (Labconco Instruments; Model: 35001) with hexane as the extraction solvent (AOAC Official Method 920.39). Total ash content was determined by a dry ashing method (AOAC Official Method 942.05), by placing samples for 1 hour at a temperature of 600°C in a muffle furnace (Thermolyne Furnace Model 62700). Total moisture content was determined by placing samples in a vacuum oven (Sheldon Manufacturing of Cornelius OR; Model 1430) for 18 hours at 70°C (AOAC Official Method 934.01).

Proximate composition analysis of the commercial feed attractants and other materials used in the pelleted diets was conducted in a similar manner described above for the Pacific hake hydrolysates, with the following modifications. The percent nitrogen was multiplied by 6.25 to obtain the percent crude protein content for the fishmeal (Higgs *et al*., 2006), while conversion factors of 5.84 and 6.64 were used for corn gluten meal and soybean meal, respectively (Fujihara *et al*., 2008; Morr, 1981). Lipid content of the test feed attractants were determined using the chloroform and methanol procedure of Bligh and Dyer (1959).

4.1.6 Size exclusion chromatography

Size exclusion chromatography on a Sephadex G-25 column was used to characterize the molecular size profiles of each of the four Pacific hake fish hydrolysates and three commercial feed attractants.

Prior to chromatography, samples were first defatted using hexane extraction with a Goldfish apparatus (AOAC Official Method 920.39) and dried in a vacuum oven (AOAC Official Method 934.01). Samples were dissolved in buffer (50 mg/mL in 0.01M phosphate buffer at pH 7.0), then filtered through 25 mm Acrodisc syringe filters with HT Tuffryn membrane (145 µm thickness and 0.45 µm pore size, Pall Corporation, New York) to remove insoluble matter. Pacific hake hydrolysates were completely soluble, while screen 1 and 2 were 75% soluble and the krill meal was only 40% soluble. Solubility was estimated by filtering out and weighing the insolubles from the solution and comparing it to the weight of the initial product.

Size exclusion chromatography was performed at a flow rate of 1 mL/min at ambient temperature, 22ºC. The samples dissolved in 1 mL of 0.01 M phosphate buffer, were applied to a 130 mL volume glass column (1.5 cm diameter and 75 cm length) containing Sephadex G-25

pre-equilibrated with 0.01 M phosphate buffer at pH 7.0 with a bed volume of 124 mL. The ultraviolet absorbance of 3 mL fractions was monitored at 214 nm and 230 nm for peptide bonds and at 280 nm for aromatic amino acid residues.

Molecular weight markers consisting of 1 mg/mL of phenylalanine (MW: 226.24 Da), vitamin B_{12}: cyanocobalamin (MW: 1355.38 Da), and hen egg white lyzozyme (MW: 14,300 Da) were run through the column before and after chromatography of the samples. The standards eluted at 54 mL (fraction 18) for lysozyme, 99 mL (fraction 33) for Vitamin B_{12}, and 120 mL (fraction 40) for phenylalanine. The size exclusion chromatography column was rinsed and stored with 0.05% sodium azide in phosphate buffer between each sample run and day, to prevent microbial growth in the resin.

4.1.7 Amino acid analyses

All four Pacific hake fish hydrolysates (ALC1, ALC4, FLA1, and FLA4) and three commercial feed attractants (krill meal, screen 1, and screen 2) were analyzed for their amino acid compositions. The freeze dried Pacific hake fish hydrolysates and the commercial feed attractants were first defatted, using the hexane extraction method (AOAC Official Method 920.39), desalted using a Micro Acilyzer S1 (Astom Corporation, Tokyo 105-8429), and then vacuum dried (AOAC Official Method 934.01) before being sent for amino acid analysis by the Advanced Protein Technology Centre (University of Toronto, Hospital for Sick Children).

The amino acid compositions of the feed attractants were analyzed using a Waters Pico-Tag System and reverse phase high performance liquid chromatography (Bidlingmeyer *et al*., 1984; Cohen and Strydom, 1988; Heinrikson and Meredith, 1984). For the total amino acid analysis, samples were dried in pyrolyzed borosilicate tubes in a vacuum centrifugal concentrator.

Samples were hydrolyzed for 24 hours using vapor phase hydrolysis under a pre-purified nitrogen atmosphere with 6 N hydrochloric acid and 1% phenol. The acid was then removed from the system by vacuum and the hydrolysates were washed with redrying solution (methanol triethylamine) followed by pre-column derivatization at room temperature with phenylisothiocyanate (PITC) to create phenylthiocarbamyl (PTC) amino acids. All primary and secondary amino acids are derivatized to very stable derivatives that can be detected at the picomole concentration level (APTC, 2009). Samples for free amino acid analysis did not undergo acid hydrolysis.

4.2. Palatability trial

4.2.1 Feed formulation and production

Krill meal, anchovy oil, and fish feed raw ingredients were all purchased from EWOS Canada. The "Nutra Fry" commercial juvenile Chinook salmon feed was purchased from Skretting Canada (Table 4.2). Based on the measured proximate compositions (data not shown) for the main fish feed ingredients (anchovy meal, soybean meal, corn gluten meal, and wheat flour), the feed formulations and steam pelleting of the fish feed were conducted by Dr. David A. Higgs, Department of Fisheries and Oceans/University of British Columbia: Centre for Aquaculture and Environmental Research, West Vancouver, BC, CAER (Table 4.3). The feed was steam pelleted to a size of 1.5 mm in diameter and 2 mm in length. Variations in specific particle size and color of feed pellets that could have affected the feeding behavior of the salmon (Mearns, 1986; Smith *et al*., 1995) were minimized by discarding any feed pellets that were exceptionally different.

The feed attractants and protein sources employed in the seven diets for the palatability feeding trial are shown in Table 4.4. The Pacific hake hydrolysates that were used consisted of the 4 hour Alcalase (ALC4) and Flavorzyme (FLA4) hydrolysates. These two attractants were selected over

their 1 hour hydrolysis counterparts because they provided a greater degree of hydrolysis yielding shorter chain peptides and free amino acids, which have been found in many studies to be important for fish taste (Sutterlin and Sutterlin, 1970). The commercial feed attractants consisted of a commercial krill meal and two experimental feed attractants, screen 1 and screen 2. The feed attractants were applied and evaluated on a soy-based fish feed that contained soybean meal at 20% of the dietary dry matter. These results were compared with a soy-based fish diet without an attractant, i.e. the soybean meal negative control, and a non-soy-based fish diet without an attractant, i.e. the fishmeal positive control (Table 4.4).

Feed attractants were added at 2% of the total feed weight (wet basis) into the anchovy oil top coating, which was 10.9% in the fishmeal positive control diet and 10.8% in the soybean meal test diets. Less supplemental anchovy oil was used in the fishmeal positive control diet (total = 13.6%) than in the soy-based diets (total = 15.1%) because the former diet contained higher levels of fishmeal, which contained more lipid than in soybean meal. Therefore, to equalize the total lipid contents in the two types of diets, less anchovy oil was required in the fishmeal based diet. The foregoing supplemental feed attractant concentrations have surpassed the minimal concentrations required to create a reaction from the fish taste receptors (Kasumyan and Doving, 2003). Minimal thresholds of feed attractants for salmonids are usually found within the 10^{-2} to 10^{-4} M range (Kasumyan and Doving, 2003).

In the test diets, soybean meal was included at 20% of the dietary dry matter and soybean protein also comprised 20% of their total protein content. The soybean meal protein replaced 24.5% of the protein derived from the fishmeal in the control diet. The fishmeal in the control diet was higher in protein content than soybean meal. Therefore, the two ingredients could not be substituted at a one to one ratio. Hence, in order to maintain equivalent protein levels in all diets

less fishmeal was removed from the control diet than soybean meal was added to the soy-based diets. Consequently, in the soybean meal diets, a portion of a low protein filler ingredient (wheat flour) was removed to make room for the soybean meal inclusion (Table 4.3).

The control diets supplemented only with anchovy oil contained slightly less protein (3.7 g/kg) than the rest of the diets. It is unlikely that the minor inclusion of extra protein in the test diets containing the feed attractants exerted any growth enhancement since all diets were high and almost equal in protein content. Alpha cellulose (2%) was added in place of a feed attractant for the soybean meal negative control diet. Alpha cellulose is a non-nutritive bulk material that does not have any effect on fish palatability (Higgs *et al*., 2006). The oil coating was then manually applied to the feed, and mixed to a homogenous state using a shaking tray. The different dietary treatments were prepared on the same day to maintain consistency. All diets were stored in airtight containers at 5°C in the dark to minimize oxidation and spoilage.

4.2.2 Saponin analyses

The seven steam pelleted diets were analyzed for saponin content at the POS Pilot Plant Corp. in Saskatchewan. The two base diets were analyzed; the fishmeal positive control diet used as the base for diet 1 and the soybean meal negative control diet used as the base for diets 2 through 7. The saponin isomers were extracted from the steam pelleted diets using methyl alcohol and then hydrolyzed with sulfuric acid (Taylor *et al*., 2000; Taylor *et al*., 1997). Nine saponin isomers (smilagenin, sarsasapogenin, diosgenin, tigogenin, yamogenin, neotigogenin/beta-sitosterol, yuccagenin, gitogenin, and neogitogenin) were separated and measured using a Hewlett Packard 5890 gas chromatograph with a HP-5 capillary column (Taylor *et al*., 2000; Taylor *et al*., 1997).

4.2.3 Mineral analyses

Trace mineral and metal contents of each of the seven diets were also analyzed and quantified. Samples were analyzed by Bodycote Testing Group, Norwest Labs in Surrey, British Columbia. Samples were digested using the reference method US EPA 3050B based on the U.S. Environmental Protection Agency Test Methods for Metals and Trace Elements (Pekey *et al*., 2004). One gram of the sample was digested with concentrated nitric acid and hydrogen peroxide at 95ºC. Twenty-nine trace minerals and metals were measured by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) based on the reference method US EPA 6010B (Pekey *et al*., 2004).

4.2.4 Feeding trial set up

The palatability feeding trial for juvenile Chinook salmon assessed the effectiveness of five different feed attractants compared to the positive and negative control diets. Seven different diets were produced and tested on triplicate tanks (groups) of fish resulting in use of twenty-one tanks.

The juvenile Chinook salmon were obtained from Spius Creek Hatchery in Merritt, British Columbia. The fish were obtained in July 2006, which falls into the optimal time period, from March to October, for feeding salmon. The salmon were a mixed, summer Nicola River stock with a brood year of late 2005 and a ponding date of April 2006. At the time of pick up, the juvenile Chinook salmon were less than 5 g each and maintained on 100% Cypress Creek water. Three thousand fish were transferred, into a 2000 L tank on a truck and then they were transported to CAER. During transportation, the total dissolved oxygen levels in the water and the water temperature were monitored hourly and controlled with an oxygen tank and bags of

ice. The water temperature was maintained at 10ºC to 15ºC and the total dissolved oxygen percent saturation was maintained at 90% to 99%.

Upon arrival, the salmon were placed into 4 disinfected 1100L tanks (approximately 750 salmon per tank) that were each supplied with running Cypress 50/50 creek water and well water. The flow rate of water into each of the tanks was maintained at 4 to 6 L per minute. The fish underwent a 30-day acclimation and growth period in the 1100 L tanks, where they were fed a commercial Skretting Nutra Fry diet (1.2mm pellets) four times daily to satiation. The water was adjusted to 100% (10-11ºC) well water 4 days before the fish were transferred to the disinfected 150 L digestibility tanks.

On day 30, the salmon in all of the 1100 L tanks were starved for an 18 hour period prior to being measured and transferred. Fish were initially sedated for about 15 minutes with clove bud oil (0.4 ppm; Hill Tech Canada Inc.). Then they were fully anaesthetized in aerated tricaine methane sulfonate (MS 222 buffered with sodium bicarbonate; Syndel Laboratories Ltd., Vancouver, BC, Canada) at a dose of 50 mg/L of water. Initially, 100 fish were weighed individually to determine their mean weight ± one standard deviation. Subsequently, all remaining fish were individually weighed and measured, and then those that were within the range of the mean weight ± one standard deviation were transferred in groups of 20 into each of the 21 150 L digestibility tanks until each contained 80 fish. Thereafter, the fish in the 21 tanks were acclimated to the experimental conditions for 14 days and during this time they were fed with the same Skretting Nutra Fry diet four times daily. The remaining stock fish were transferred into an outdoor 4000 L fiberglass tank and fed twice daily on the commercial Skretting Nutra Fry diet to apparent satiation.

Prior to being sampled and measured for their individual weights and lengths, all fish in each group were starved for an 18 hour period. The salmon were weighed and measured individually on days 0 and 35 of the study following a dual anesthetic procedure involving the use of a clove bud oil solution, followed by a buffered MS 222 solution, using the conditions previously described.

On day 0, 75 fish in each tank were returned to their original tanks for the palatability feeding trial after being weighed and measured. Five fish from each tank were euthanized by a quick blow to the head and stored in vacuum sealed bags in a -18ºC freezer for future *Kudoa* spore quantification. On day 30, 70 of the fish in each tank were returned to their original tanks for the 7-day fecal collection period after being weighted and measured. Another 5 fish from each tank were euthanized and stored in vacuum sealed bags in a -18ºC freezer for future *Kudoa* spore quantification. Upon completion of the entire 35-day feeding trial and 7-day fecal collection period, all fish were placed in the 4000 L stock tank and maintained for future research projects. The distribution of fish is shown in Table 4.5.

Throughout the 35-day feeding trial period, the 150 L digestibility tanks were equipped with flowing, aerated water (96-97% dissolved oxygen) at approximately 4 to 6 L per minute to ensure optimal water quality. A black plexiglass cover was placed over each tank to minimize auditory and visual disturbances and the hinged cover was only lifted during feeding times of approximately 10 minutes, 3 times a day (Oikawa and March, 1997). The opacity of the tanks also helped minimize any visual disturbances to the fish in the tanks. To minimize stress, the water flow rate was never stopped, even during feedings.

To minimize potential bias, all diets were randomly coded such that the feeder was blind to treatment. As well, block randomization was applied to the cycle of feeding to ensure that fish in triplicate tanks were not fed in sequential order.

The Chinook salmon in each group were hand fed 3 times daily at 9:00 am, 12:00 pm, and 3:00 pm. Feed pellets were delivered as quickly as the fish would consume them and this was continued until the point of apparent satiety was reached. It would be impossible to judge the point of true satiation within a tank consisting of 75 fish. Therefore, apparent satiation was chosen and judged to be attained when 75% of the pellets dispensed at any given time fell to the bottom of the tank and into the drain. The amount of time it took to feed one tank of fish varied from 10 to 20 minutes depending on the diet. The palatability of the diet was assessed by determining the frequency with which the pellets were exchanged between fish (Sutterlin and Sutterlin, 1970). The number of uneaten pellets was counted and this number was multiplied by the mean air-dry weight of the pellets for a given diet to provide an estimate of feed wastage. This, in turn, was deducted from the daily ration dispensed to give an estimate of the actual ration consumed by each group per day.

All equipment used in the feeding and sampling of fish were sanitized with chlorine (50 mg of hypochlorite/L of water) or heated to 90ºC to kill the *Kudoa* spores from the feed and to prevent cross contamination.

The feces were also collected post feeding during the last 7 days of the study using the Guelph system of fecal collection as described by Hajen *et al*. (1993). The specially designed digestibility tank complex has a fecal collection column affixed to each tank to enable collection of the feces daily. After the last feeding of the day, all fecal and feed residues were brushed and

cleaned out of the tank and drain system. The feces were obtained from the fecal collection column the following morning. Fecal matter was centrifuged at 10,000g for 30 minutes at 5ºC to remove the supernatant, which was the excess water. The centrifuged fecal material was frozen overnight, freeze dried the following day and ground to a fine powder for spore extraction to determine the spore absorption percentage. During the 7-day fecal collection period, the salmon were fed to apparent satiation 4 times daily.

4.2.5 Fish health

A fish health checklist (Table 4.6) was used to check the health of all fish each day and whether they were able to continue with the palatability feeding trial. Fish health was assessed and monitored closely throughout the feeding trial to ensure health standards were met. The drains of the tanks were scrubbed clean with gloved hands approximately every 5 days. This ensured that no adverse bacterial or mold growth would occur in the tanks. The water environment consisting of total dissolved oxygen, water temperature, and total dissolved solids was checked daily to ensure consistency and to maintain a safe level for the fish. Equivalent water temperatures between tanks were especially important to confirm since temperature has a major effect on appetite of fish (Kasumyan and Doving, 2003). Fish behaviour, color, and signs of disease were monitored and the intensity of each was recorded. When a fish was unable to continue with the program by having an intensity level of D in any of the three categories, it was removed from the tank to prevent contaminating the other fish and then the fish was euthanized by a quick blow to the head.

4.2.6 Feed attractant performance parameters

The initial and final weight of each container of feed was recorded for each feeding of each day and the difference in weight was calculated. Daily feed wastage for each group was then

deducted from the total weight of feed dispensed to provide an estimate of the actual ration consumed by each group of fish (Helland *et al*., 1996). Fish mortality was recorded daily for each group to help determine the dry feed intake in grams for each individual fish per day (n = 3 estimates per diet treatment). Feed consumption was also expressed in relation to the geometric mean body weight of each group during the trial according to the equation used by Richardson *et al* (1985). In addition, the weight gains, specific growth rates, feed efficiencies, and the percent survival rate of fish within each tank were calculated (Raven *et al*., 2005). The rate of how long it took for the fish to acclimate to their respective diets was also measured. The degree of acclimation was based on the daily feed intake trend (grams/day) for days 1 to 15 (slope=m). Days 16 to 35 were not used because there were no significant differences within or between diets indicating the acclimation period had ended and feed intake had reached a plateau.

4.2.7 Kudoa spore extraction and quantification

Kudoa spore extraction and counts were conducted for every Pacific hake used in the FPH production (41 fish in total), and on the Chinook salmon fillets collected from the palatability feeding trial on days 0 and 35. Samples from the nape region of the fish (6 x 4 x 4 cm) were removed after overnight thawing of the fish, followed by the removal of the skin and bones (Dawson-Coates *et al*., 2003). The muscle samples were digested with trypsin (T7409 from Sigma-Aldrich Canada Ltd, Oakville, ON) and the spores were extracted following the method described by Samaranayaka *et al.* (2006). The trypsin solution was prepared by dissolving $4x10^{-3}$ g of dried porcine trypsin enzyme into 10 mL of a phosphate buffer saline solution (pH 7.4). This 1:25 trypsin:fish ratio was determined in preliminary studies to be sufficient for digesting $1x10^{-1}$ g of fish mince to release the spores. To maximize the effectiveness of the enzyme activity, the solution was prepared just before its use. All samples were analyzed in duplicate.

The spore extraction process for whole fish, commercial feed attractants, and fecal matter was similar to that described above for muscle tissue. An additional filtration step was used after the trypsin digestion, where the minced fish solutions were placed in a Buchner vacuum filtration system with Whatman filter paper with 20 μm pore size, sufficient to filter out any impurities while allowing the spores (5 to 10 μm in size) to pass through (Moran *et al*., 1999). The filtered liquid was then centrifuged at 17,000 *g* for 15 minutes at 4ºC. Subsequently, the supernatant was removed and the pellet was re-suspended in 1 mL of phosphate buffered saline (PBS) solution and mixed with a vortex. Thereafter, the slurry was placed gently into 15% and 30% Percoll© solutions in a 15 mL falcon tube and centrifuged at 17,000 *g* for 15 minutes at 4ºC. The filtration gradient was created by layering 2.5 mL of 15% Percoll© solution on top of 2.5 mL of 30% Percoll© solution. After centrifuging, the supernatant was discarded leaving behind 1 mL of spore suspension. The samples, which were transferred to 2 mL Eppendorf plastic vials, were frozen at -18ºC until further analysis.

The four Pacific hake fish protein hydrolysate feed attractants did not require any extraction procedure as they had already been filtered during production and consisted only of soluble compounds. Therefore, they were only re-suspended in 1 mL of buffer before further spore quantification analyses.

Enumeration of spores in the prepared extracts or hydrolysate solutions was conducted, in triplicate, as previously described (Samaranayaka et al., 2006; Meng and Li-Chan 2007). Prior to analysis, frozen samples containing the spores in the Eppendorf plastic vials were thawed for 5 to 10 minutes at room temperature, 20ºC, and mixed well. Aliquots (10 μL) of spore extract or hydrolysate solution were distributed onto each side of a haemocytometer. The spores were counted under a 20 x objective lens (200 x magnification including the 10 x occular lens) and the

average of the eight regions was recorded. Dilutions of 2, 10, or 100 times with PBS solution were performed as necessary to ensure counts in the range of 20 to 50 spores per area. *Kudoa paniformis* and *thyrsites* were counted and recorded separately.

4.2.8 Statistical analyses

The results for the palatability parameters were subjected to one-way analysis of variance between the different dietary treatments and two-way analysis of variance for the comparisons of different dietary treatments and time. This was followed by Tukey's multiple comparison post hoc tests for individual treatment comparisons ($P < 0.05$) where appropriate. The statistical analyses were performed using SPSS for Windows (Standard Version, Build 10.0.1 SPSS Inc.). Statistical significance is reported at the 5% level unless otherwise noted.

5.0 RESULTS

5.1 Feed attractant characterization

5.1.1. Production of hydrolysates from Pacific hake

Whole Pacific hake hydrolysates produced with Alcalase yielded a significantly higher FPH recovery than that of hydrolysates produced with Flavorzyme (Table 5.1). However, there were no differences in the FPH recovery between the hydrolysates produced with the same enzymes at different hydrolysis times.

5.1.2. Proximate composition

Proximate analyses were expressed on a wet weight (as is) basis to mirror the use of ingredients in the fish feed industry, which were also on a wet weight (as is) basis, as shown in Table 5.2. The Alcalase produced hydrolysates had the highest crude protein content followed by the Flavorzyme produced hydrolysates and the commercial feed attractants. Not surprisingly, the Pacific hake hydrolysates contained the least amount of fat because of the removal of fat during the production stage. Ash was also lower in the Pacific hake hydrolysates due to the use of deionized water and not having pH adjusted during production. Screen 2 commercial feed attractant had the lowest amount of crude protein, but contained the highest concentration of moisture, giving it a fudge-like consistency. The sum of percent crude protein, fat, moisture and ash in each of the Pacific hake hydrolysates was less than 100%, indicating that other compounds may be present in the hydrolysates, such as carbohydrates, nucleotides, nucleosides, and amines (Kasumyan and Doving, 2003).

5.1.3 Amino acid profile

The four Pacific hake hydrolysate feed attractants had similar total amino acid profiles (Table 5.3). All four hydrolysates were produced from the same batch of minced whole Pacific hake,

which may contribute to the similarity in total amino acid profiles after proteolysis to produce the hydrolysates. They all contained high levels of aspartic acid or asparagine, glutamic acid or glutamine, leucine, and lysine. There were also some similarities between the Pacific hake hydrolysates and the commercial attractants. Aspartic acid or asparagine, glutamic acid or glutamine, leucine, and lysine were the dominant amino acids in the commercial attractants as well. The screen 1 and screen 2 feed attractants also had high concentrations of glycine, histidine, and taurine and less tyrosine and cysteine compared to the Pacific hake hydrolysates. The krill meal had higher levels of glutamic acid or glutamine, tyrosine, arginine, valine, methionine, isoleucince, leucine, and phenylalanine compared to all other feed attractants.

The profiles of free amino acids (Table 5.4) show that Flavorzyme hydrolysates contained a greater amount of free amino acids than the Alcalase hydrolysates and the commercial attractants. This could be because Flavorzyme has exopeptidase and endopeptidase activity (Khan and Li-Chan, 2008). The Alcalase hydrolysates showed a similar trend in their free amino acid profiles, but each of the amino acids was at lower concentrations than in the Flavorzyme hydrolysates. For the majority of the free amino acids, there were no differences between the 4 hour hydrolysates and the 1 hour hydrolysates.

The krill meal contained very low levels of free amino acids (Table 5.4). Screen 1 and screen 2 feed attractants had similar free amino acid profiles; both showed higher concentrations of histidine, taurine, and proline in comparison to the Pacific hake hydrolysates. However, in comparison to the latter they also generally had very little asparagine, serine, glutamine, arginine, tyrosine, cysteine, and tryptophan.

When each of the free amino acid concentrations in the test products was expressed as percentages of their respective totals, it can be seen that all of the histidine and taurine for the screen 1 and 2 attractants was in the free form. A high percentage of taurine was also in the free form in all the fish protein hydrolysates and feed attractants. Cysteine was similar in having a higher percentage present in the free form in all fish hydrolysates and feed attractants. In general, a high percentage of free amino acids was present in the Flavorzyme fish hydrolysates, including serine, taurine, arginine, threonine, alanine, tyrosine, valine, methionine, cysteine, isoleucine, leucine, phenylalanine, and lysine.

5.1.4 Size exclusion chromatography

The feed attractants were analyzed using size exclusion chromatography to separate their compounds based on molecular size. Eluent fractions (3 mL/fraction) were scanned at 214 nm (Fig 5.1) and 230 nm (Fig 5.2) to monitor peptide bonds and at 280 nm (Fig 5.3) to monitor aromatic compounds. Each fraction number in the x-axis represents a 3 mL volume. The absorbance for each sample was shown on the y-axis. For example, the void volume of 54 mL point was represented by the 18 point on the x-axis.

As shown in Figure 5.1, monitoring of the eluent at 214 nm indicated that in general, Flavorzyme hydrolysates had higher concentrations of lower molecular weight compounds, whereas Alcalase had a higher concentration of larger molecular weight compounds. The sizes of the eluted compounds were calculated from a graph of molecular weights and elution volumes of the known molecular weights of the three standards using a logarithmic molecular weight vs mobility curve. After the curve was established, molecular weights of the compounds were estimated based on where they were eluted on the curve. When compared to the 4 hour Alcalase hydrolysate, the 1 hour Alcalase hydrolysate had a higher level of large molecular weight

compounds in the regions of 14,300-5,080 Da (fraction 18-24) and smaller amounts of lower molecular weight compounds in the 480-165 Da (fraction 36-40) and <226.24 Da (fraction 56-64) region. When comparing the two Flavorzyme hydrolysates, the 1 hour Flavorzyme hydrolysate had a higher amount of large molecular weight compounds in the 24,200-11,100 Da (fraction 16-20) region and the 4 hour Flavoryzme hydrolysate had higher amounts of compounds in the 7,500-100 Da (fraction 22-44) and <226.24 Da (fraction 60-64) regions. These smaller molecular weight compounds could have been free amino acids or salts.

In Figure 5.2, with a 230 nm scan, similar results were found with a higher level of larger molecular weight compounds in the 1 hour hydrolysates when compared with their 4 hour hydrolysate counterparts. The 1-hour Alcalase feed attractant had a higher level of compounds in the 14,300-5,080 Da (fraction 18-24) region compared to the 4-hour Alcalase feed attractant. The 1-hour Flavorzyme feed attractant had a higher level of compounds in the 24,200-11,100 Da (fraction 16-20) region compared to the 4 hour Flavorzyme feed attractant.

The differences between hydrolysates were not as pronounced for the aromatic compounds monitored by absorbance at 280 nm (Figure 5.3) compared to the results shown in Figures 5.1 and 5.2 reflecting the peptide bond. However, there were still some differences. The 4-hour Alcalase hydrolysate had a higher amount of aromatic compounds than the 1-hour Alcalase hydrolysate, especially in the <226.24 Da (fraction 40-45) and <226.24 Da (fraction 54-60) regions. The same can be said for the 4-hour Flavorzyme hydrolysate compared to the 1-hour Flavorzyme hydrolysate in the regions of <226.24 Da (fraction 42-44). The 1-hour Flavorzyme hydrolysate also had a greater level of aromatic compounds in the 14,300-11,100 Da (fraction 18-20) region compared to the 4-hour Flavorzyme hydrolysate.

The two commercial feed attractants screen 1 and screen 2 (Figures 5.4-5.6), exhibited similar size exclusion chromatography profiles, even though their physical appearances and consistencies varied greatly. The screen 1 attractant was a very fine white powder, whereas screen 2 was a brown, high moisture, viscous substance. One marked difference between the two was that screen 1 had a higher amounts of smaller molecular weight compounds in the 480-165 Da (fraction 36-40) and <226.24 Da (fraction 46-50) regions in the 214 nm scan. The 230 nm scan provided similar results with again, screen 1 showing a higher amounts of smaller molecular weight compounds in the 480-165 Da (fraction 36-40) and <226.24 Da (fraction 46-50) regions. For both the 214 nm and 230 nm scans, screen 1 also had a higher concentration of larger molecular weight compounds in the 20,000-13,500 Da (fraction 17-10) region. As for the aromatic amino acids scanned at 280 nm (Figure 5.6), the screen 1 feed attractant had higher levels of aromatics.

Differences were seen in terms of the position of the peaks among the four Pacific hake hydrolysates. However, the differences between the Pacific hake hydrolysates and the commercial feed attractants were much more pronounced. The size exclusion profiles of the commercial feed attractants had sharper peaks, whereas the profiles for the Pacific hake hydrolysates had much broader peaks that could have been due to a broad profile of different sized peptides and free amino acids associated with the proteolysis of the entire hake fish. In addition, the commercial feed attractants did not contain small molecular weight compounds below the 30 Da (fraction 50) mark. It is possible the commercial feed attractants may have already undergone fractionation or purification to remove smaller molecular weight compounds.

5.2 Saponin and trace mineral profiles of formulated diets

As shown in Table 5.6, out of the nine saponins measured, only neotigonenin/beta-sitosterol was found in both soybean containing and fishmeal only diets. The saponin content was slightly higher in the soybean meal negative control diet, which contained 20% soybean meal, than the fishmeal positive control diet, which did not contain any soybean meal. Neotigonenin/beta-sitosterol was expected to be in the soy diets because it is a component of soybean meal (Normen *et al*., 2006). Its presence in the fishmeal diet without soybean meal may be a result of the inclusion in all diets of corn gluten meal and wheat flour, which can contain trace amounts of corn oil and wheat germ (Normen *et al*., 2006). These two ingredients can be potential sources of neotigonenin/beta-sitosterol (Normen *et al*., 2006).

Table 5.7 indicates that the major differences in the dietary mineral profile were between the fishmeal positive control diet, which did not contain soybean meal, and the rest of the diets that all contained 20% soybean meal. The fishmeal positive control diet had less aluminum, molybdenum, nickel, potassium, titanium, zirconium, and thallium. It also contained higher levels of manganese and selenium when compared to the other six diets. Most of the trace mineral profiles were similar for the six soybean meal diets because the 2% feed attractant difference between diets did not lead to any significant differences in the mineral profiles of the diets. However, the sodium level was higher in the screen 1 and 2 diets compared with the rest of the soy diets. The screen 1 and Alcalase diets had higher levels of cobalt as well.

5.3 Palatability Trial

5.3.1 Feed Attractant Performance

Feed intake was measured for salmon in each of the triplicate tanks in relation to each type of diet. Feed intake for each fish was calculated as a daily average (Figure 5.7) and as the total amount consumed in the 35-day feeding trial on a dry basis (Figure 5.8). The two graphs show similar trends, with the fish fed the fishmeal positive control, basal diet having significantly higher feed intake than the fish fed the six soy-based diets. For the fish within the six soy-based diets, those fed the krill meal diet and the screen 1 diet displayed a significantly higher feed intake than the fish fed the soybean meal negative control diet.

Feed efficiency ratio is the ratio of the amount of body mass increase in the fish to the amount of dry feed consumed. Therefore, a ratio of 1 indicates that for every 1 g of dry feed consumed, there will be 1 g of body mass increase. Figure 5.9 shows that the fish fed the fishmeal positive control, basal diet had a significantly higher feed efficiency ratio than the fish fed the soybean meal negative control and other soy-based diets. However, the fish that consumed the krill meal, Alcalase, and screen 1 diets showed significantly higher feed efficiency ratios than the fish that consumed the soybean meal negative control diet.

Specific growth rate of the fish is defined as the percent increase in the fish's bodyweight or length per day. Parallel trends were observed for the specific growth rates for body weight and length (Figure 5.10). The fish fed the fishmeal positive control diet had a significantly higher specific growth rate than the fish fed the soy-based diets. Nevertheless, within the groups fed the soy-based diets, the fish fed the krill meal, Alcalase, and screen 1 diets had significantly higher specific growth rates than those fed the soybean meal negative control diet.

The actual gains in weight and length of all the fish, measured for the 35-day period, are shown in Figure 5.11, and these parameters indicate that the growth of the fish fed the fishmeal positive control diet was significantly higher than the fish fed the rest of the diets. Moreover, fish fed the diets containing krill meal, Alcalase, and screen 1 had significantly higher gains in weight and length than those fed the soybean meal negative control diet containing no feed attractant.

Feed wasted is defined as the amount of feed dispensed into the tank at one feeding corrected for feed consumed. Therefore, it is identified by the amount of feed that falls to the bottom of the tank and into the drain, unconsumed. The amount of feed wasted was measured and compared to the total feed intake of the groups fed each diet for the 35 days (Figure 5.12). The fish that ate the fishmeal positive control diet had a significantly higher amount of feed wasted than the fish fed the other six diets. Similar trends can also be seen with the combination of feed wasted and feed dispensed for the fish fed the seven diets.

Although feed wasted for the fish fed the fishmeal positive control diet was relatively higher than noted for fish fed the other diets (Figure 5.12), the total feed intake was also much higher for those fish. In fact, as shown in Figure 5.13, when the feed wasted was expressed as a percentage of the amount of feed dispensed and consumed, the percent feed wasted was significantly lower for the fish fed the fishmeal positive control diet than for most of the fish fed the other diets. In addition, the percent of feed wasted was significantly lower for the fish fed the fishmeal positive control diet, krill meal diet, Alcalase diet, and screen 1 diet when compared to the fish fed the soybean meal negative control diet.

The feed intake trend can be defined as how fast the fish adapted to the diets, or length of the acclimation period that was required before the fish began to eat the diet at maximum ration. This parameter was measured as a slope value based on the feed intake over the first few days until feed intake leveled off, and thus the higher the slope the quicker the fish adapted to the diet. Figure 5.14 shows that the fish fed the basal diet adapted significantly faster than the fish fed the soy-based diets. Further, the fish fed the krill meal and the screen 1 diets showed significantly higher adaptation values than the fish fed the soybean meal negative control and the Flavorzyme diets.

5.3.2 Kudoa Spore Quantification

The *Kudoa paniformis* count in fish mince used to produce the fish protein hydrolysates was 5.29 x 10^6 spores per gram while the *Kudoa thyrsites* count was 1.67 x 10^4 spores per gram (Table 5.7). The *Kudoa* spore count of the fish mince was reduced by 141 to 635 fold for *Kudoa paniformis* and 4 fold for *Kudoa thyrsites* after producing the fish hydrolysates (Table 5.7). Therefore, a fraction of the spores was removed during production of the fish hydrolysates. This could have been in the centrifugation step in the production of the Pacific hake hydrolysates where the insoluble and bone fractions were removed. It is also possible that the spores were removed during filtration of the hydrolysate supernatant.

Kudoa paniformis and *thyrsites* were not detected in any of the three commercial feed attractants (krill meal, screen 1, and screen 2). There were no detectable *Kudoa* spores in the fecal matter from the fish fed the fishmeal positive control diet, the soybean meal negative control diet, the Alcalase diet and the Flavorzyme diet. Neither type of *Kudoa* spores were detected in the fillets from Chinook salmon fed the soybean meal negative control diet, the Alcalase diet and the Flavorzyme diet when fish were collected on days, 0 and 35 of the palatability feeding trial. The

detection method used cannot detect spore populations below 6.25 x 10^3 spores per gram for a given sample (Meng and Li-Chan, 2007). However, the values shown in Table 5.7 are averages of 3 replicates, thereby sometimes giving values that are lower than the actual detection limit if one or more of the replicates had undetectable spore levels.

6.0 DISCUSSION

6.1 Pacific hake hydrolysates

Four Pacific hake protein hydrolysates were produced, using different enzymes and hydrolysis times, to be used as potential feed attractants. Alcalase and Flavorzyme were chosen because they were reported to give high degrees of hydrolysis when compared with Protamex, another commercial enzyme tested (Khan and Li-Chan, 2008). The choice of the enzymes was also based on other studies that produced hydrolysates with these enzymes and those hydrolysates exhibited excellent functional properties (Kristinsson and Rasco, 2000). From the four Pacific hake hydrolysates produced in the current study, two hydrolysates were chosen for evaluation as potential feed attractants in the fish feeding trial based on their degrees of hydrolysis and amino acid profiles (Khan and Li-Chan, 2008).

The hydrolysate produced by Alcalase was much more effective as a feed attractant than the hydrolysate produced by Flavorzyme. Alcalase, a proteinase from *Bacillus licheniformis*, only has endopeptidase activity, whereas Flavorzyme, a fungal protease/peptidase complex from *Aspergillus oryzae*, has both endo and exopeptidase activity (Novozymes, 2004a; Novozymes, 2004b). Therefore, Flavorzyme tends to produce more free amino acids by cleaving the terminal amino acids from peptides and Alcalase would produce more peptides. However, some studies have found that free amino acids are more effective as a feed attractant than peptides (He *et al*., 2006; Sutterlin and Sutterlin, 1970).

6.2 Amino acid profiles

The total content of amino acids ranged between 44.92 and 59.45 grams of amino acids per 100 grams of sample for the 7 hydrolysates and feed attractants (Table 5.3). By comparing the content of total amino acids for the hydrolysates with their crude protein content measured by the level of nitrogen (Table 5.2), it may be surmised that there are other nitrogen containing compounds in the samples, such as amines, amides and nucleotides (Kasumyan and Doving, 2003). There is also the possibility of carbohydrates or ash still present in the sample before the amino acid analysis.

The five dietary feed attractants used in this study were produced from different marine sources. This resulted in the feed attractants having very different free amino acid compositions. The major differences in free amino acid compositions were found between the two Pacific hake hydrolysates and the screen 1 and screen 2 commercial feed attractants (Table 5.4). The two Pacific hake hydrolysates contained higher concentrations of aspartic acid, glutamic acid, serine, arginine, threonine, methionine, valine, leucine, phenylalanine, and lysine. However, the screen 1 and screen 2 commercial feed attractants had higher concentrations of histidine, taurine, and proline.

Interestingly, two of the three free amino acids found in higher concentrations in the two commercial feed attractants were the most potent feed attractants, proline and taurine, found in several studies (Sutterlin and Sutterlin, 1970; Mearns, 1986). The Pacific hake hydrolysates contained methionine and leucine, which are two amino acids found to be effective feed attractants in other studies (Sutterlin and Sutterlin, 1970; Mearns, 1986). The Flavorzyme hydrolysates also contained higher levels of aspartic acid, glutamic acid, arginine, alanine,

valine, lysine, isoleucine, and leucine than the Alcalase hydrolysates. Thus, the Flavorzyme hydrolysates contained more free amino acids than the Alcalase hydrolysates and krill meal. The results of this study show that the fish fed the krill and Alcalase diets, with lower levels of free amino acids, had a significantly higher growth rate, feed intake and weight gain than the fish fed the Flavorzyme diet. These results contradict other studies that indicate free amino acids are more effective than peptides as feed attractants (He *et al*., 2006; Sutterlin and Sutterlin, 1970). It is quite possible that some of the free amino acids found in the Flavorzyme hydrolysates were unpalatable for salmon. In addition, combination of other compounds with amino acids can result in synergistic or antagonistic effects. For example, betaine enhanced the palatability of alanine and glycine for red sea bream (Kasumyan and Doving, 2003) whereas certain heavy metals can neutralize the effects of palatable compounds (Sutterlin and Sutterlin, 1970).

The two EWOS commercial feed attractants had similar free amino acid compositions. However, there were significant differences in terms of the effectiveness of these feed attractants. This implies that other factors, such as the presence of other attractive compounds that are not amino acids or peptides may play a part in the effectiveness of these feed attractants. The sequence and size of the peptides found within the hydrolysates or attractants could have also affected the efficacy of the feed attractants. Small differences between amino acid compositions may be sufficient to cause a change in the dietary preferences of the fish.

6.3 Size exclusion chromatography

The krill meal sample was filtered to remove insoluble compounds prior to size exclusion chromatography. Thus, not surprisingly, the krill meal size exclusion chromatograms had fewer compounds compared with the other feed attractants. The krill meal was not hydrolyzed and therefore most likely contained higher levels of proteins and lower levels of peptides and amino

acids. The higher levels of proteins could explain why the krill meal contained very little free amino acids compared to the other hydrolysates and feed attractants. It is possible that the insoluble fraction of the krill meal was insoluble proteins and chitin. The ratio of soluble aromatic compounds to peptide bonds was a lot higher in the krill meal. This could be associated with the high levels of tyrosine and phenylalanine in the total amino acid profile for krill meal.

The commercial feed attractants displayed high contents of free amino acids. Their free amino acid content was even higher than the Pacific hake hydrolysates created with the Alcalase, but were lower than the levels observed in the Flavorzyme hake hydrolysates.

The UV-absorbances of fractions from size exclusion chromatography were measured at 214 and 230 nm. These wavelengths were used to detect the peptide bonds in the fractions and to estimate the presence of peptides. Different peptides in hydrolysates can have a positive effect on feed intake for certain fish when incorporated into their diet (Aksnes *et al*., 2006b). The absorbance at 280 nm was also measured to determine the presence of any aromatic compounds, which can potentially act as feed attractants for fish. Tyrosine and phenylalanine, both aromatic amino acids, have stimulatory effects in certain fish, such as rainbow trout (Adron and Mackie, 1978; Hara *et al*., 1999).

Most of the peaks in the size exclusion chromatograms represented large molecular weight compounds in the 14,300 to 600 Da (fraction 18-35) region, which are likely associated with the different proteins and peptides found in the feed attractants and hydrolysates. The commercial feed attractants had sharper peaks in this large molecular weight region indicating that they consisted mainly of larger peptides with fewer size differences. Absorbance was also detected in the amino acid size region of 200 to 75 Da (fraction 40-45) at all three wavelengths. This was

seen with the four Pacific hake hydrolysates, with a higher absorbance found in the 4 hour hydrolysates than their 1 hour hydrolysate counterparts. This matches the free amino acid profiles indicating that the 4 hour hydrolysates contained higher levels of free amino acids.

6.4 Saponin profile

The total level of saponins found in the diets was relatively low with little differences between the basal diet and the soy test diets which contained 20% soybean meal. The only saponin detected was neotigonenin/beta-sitosterol at a concentration of 0.12 mg/g in the basal fish feed pellets and 0.18 mg/g in the fish feed pellets containing the soybean meal. If the soyasaponins in the diet were from the soybean meal alone, then the concentration of the soyasaponins in the soybean meal used would be 0.9 mg/g. This concentration was much lower than what was found in other studies, where soybean meal and soy flour contained between 0.43 to 0.67% soyasaponins (Bureau *et al*., 1998). This is most likely due to the variations in how the soybeans were processed and different soy varieties. Different soybean protein products, like soybean meal, soy protein concentrate, and soy protein isolate contain different levels of soyasaponins (Zhou *et al*., 2005). The level of saponins found in soybean meal can even differ depending on where and how the soybean meal is made and what specific type of soybean meal, such as defatted, full fat, or low oligosaccharides soybean meal is used (Zhou *et al*., 2005). The reduction in the palatability of the diet is strongly linked with the level of soyasaponins found in the soybean meal diets (Bureau *et al*., 1998).

Various and inconsistent manufacturing practices can result in products with varying proximate compositions and different levels of micronutrients and antinutrients. This can also be seen with the production of meat and bone meal, and poultry by-product meal (Goda *et al*., 2007a).

The level of saponins found the fishmeal positive control diet and the soybean meal negative control diet were similar, but the level of feed intake and growth between the fish fed these two diets was significant. This could imply that other antinutrients or compounds other than saponins could have acted as deterrents and thereby negatively affected the palatability of the diets and growth of the fish. These could include, but are not limited to protease inhibitors, phytic acid, and lectins (Drackley, 2000; Gatlin *et al*., 2007). Another possible reason for the differences in feed intake between groups may have been the presence of other attractive compounds within the fishmeal used to produce the fish feed pellets. The fishmeal positive control diet contained 20% more fishmeal than the soybean meal negative control diet, which could have resulted in a higher concentration of attractive compounds in the diet.

6.5 Trace mineral profile

A series of heavy metals have been shown in other studies to act as blocking agents towards the facial and palatine nerves of the fish. This can have a negative effect on the taste response systems in different salmon species (Sutterlin and Sutterlin, 1970). The minerals most effective as blocking agents were mercury and lead (Sutterlin and Sutterlin, 1970). Certain chloride salts, such as potassium and sodium were stimulatory for certain salmon species (Sutterlin and Sutterlin, 1970). The fishmeal positive control diet in this study had lower levels of potassium compared to the six soy-based test diets. The soybean meal diets contained higher levels of aluminum and nickel. The basal fish feed diet contained higher levels of cobalt, iron, manganese, and selenium. However, these trace minerals have not been mentioned as having an effect on fish diet palatability.

6.6 Feeding trial set up

Many precautionary steps were taken in setting up the fish tanks and throughout the feeding process. The number of fish placed into each tank was ensured to exceed a certain level because the size of the group can have an influence on their feeding behaviour and thus affect the satiation level of the fish. Seventy-five fish per tank was found to exceed the level necessary to achieve maximum satiation level for the fish (Ishiwata, 1979). Only fish of similar size and weight were used in the study because large differences in fish size in one tank can affect the level of acceptance and aggressive behavior within the tank (Reig *et al*., 2003). Fish feed was also handled with rubber gloves and dispensed into the fish tanks with a plastic spoon to avoid contaminating the feed. Salmon can be very sensitive to the dilute concentrations of human skin secretions (Brett and MacKinnon, 1954). Fish were fed three times a day, since this has been determined to be the ideal feeding frequency for optimal growth of juvenile salmon in the weight range that was used (Barrows and Hardy, 2001). Feeding times were consistent for every day of the feeding trial because the stability of the feeding times may influence the acceptance of the diet even more so than differences in composition between the diets themselves (Reig *et al*., 2003).

6.7 Feed attractant effectiveness measurements

The results for juvenile Chinook salmon fed the soy-based diets were not unexpected. Other studies have reported negative effects of soybean meal inclusion in the diet of Chinook salmon with respect to their growth and feed intake (Bureau *et al*., 1998). Therefore, it was expected that the fish fed the positive control diet would surpass the fish fed the soy-based diets in all fish performance parameters.

However, even with feed attractants applied, the fish fed the soy-based diets were still significantly lower in the majority of the fish performance parameters. The one exception was percent of feed wasted, where the fish fed the diets coated with krill meal, Pacific hake hydrolysate produced from Alcalase, and screen 1 attractants did not show significant differences when compared with the fish fed the fishmeal positive control diet. The diets formulated for the feeding trials did not contain supplemental lysine because it was not limiting. Therefore, it is possible that the positive control diet contained higher levels of lysine because of its higher level of fishmeal. This could be a possible reason that the fish fed the positive control diet performed better because of lysine possibly having attracting effects (Brown, *et al*., 1997). Another possibility is that soybean meal can contain some protease inhibitors that were not completely inactivated by heat treatments during processing, which can lower its nutrient availability (Drackley, 2000).

The screen 2 feed attractant was not as effective as the other commercial feed attractants. Possibly, this was due to its high moisture content. All feed attractants were added to the fish feed pellets on a wet weight basis. The high moisture content would have led to a lower concentration of protein, peptides, amino acids, and other palatable compounds added as a top coating to the feed pellets.

The majority of the feed attractant parameters were directly linked with each other. Namely, fish that displayed a higher feed intake would most likely have a higher weight gain and specific growth rate. The higher feed efficiency ratio found in the fish fed the fishmeal positive control diet could most likely be attributed to the many antinutrients found in soybean meal that can inhibit proper nutrient absorption (Bureau *et al*., 1998). The higher acclimation trend found in the fish fed the fishmeal positive control diet may have been due to the presence of feeding

deterrents found in the soybean meal present in all the other diets, which was also the cause for the lower feed intake (Bureau *et al*., 1998). The main difference between the fishmeal positive control diet and the other six test diets was the presence of soybean meal in the test diets. Therefore, it is quite possible that the soybean meal was the main cause of the lower feed intake in the fish. Other studies have already determined that soybean meal contains high levels of soyasaponins, phytic acid, lectins, and other foreign compounds, which can be strong feeding deterrents and detrimental to fish health (Bureau *et al*., 1998).

6.8 Kudoa spore quantification

Kudoa paniformis and *thyrsites* were not detected in any of the three commercial feed attractants (krill meal, screen 1, and screen 2). Chinook salmon fecal matter was analyzed from tanks of salmon fed the fishmeal positive control diet, the soybean meal negative control diet, the Alcalase diet, and the Flavorzyme diet. No *Kudoa* spores were detected in the fecal matter. Neither type of *Kudoa* spores was detected in the fillets from Chinook salmon, collected on day 0 and on day 35, when they had been fed the soybean meal negative control diet, the Alcalase diet, and the Flavorzyme diet during the palatability feeding trial.

There were no detectable *Kudoa* spores in the commercial feed attractants. This means the ingredients used for the commercial feed attractants likely did not contain any highly *Kudoa* parasite infected marine sources, such as Pacific hake and Atlantic salmon. It might also mean that such sources may have been used but use of processing operations such as filtration may have removed spores present in the starting materials.

There were no detectable signs of the presence of *Kudoa* spores in the Chinook salmon fecal matter tested. This could simply be because the level of *Kudoa* spores found in the diet was

already below detectable limits using the Dawson-Coates *et al*. (2003) trypsin assisted *Kudoa* spore extraction method and the haemocytometer quantification method. The method was introduced by St-Hilaire *et al*. (1997) and was much more accurate than estimating the average number of pseudocysts found in the muscle tissue (Adlerstein and Dorn, 1998). The trypsin method was later improved by analyzing the spore counts from multiple muscle sites and better release of the spores from the muscle for more accurate results (Dawson-Coates *et al*., 2003). Therefore, this was the most feasible method for detecting low levels of *Kudoa* spores for this study. However, the feed attractants used already contained *Kudoa* spores at minimally detectable levels (10^3 and 10^4) or undetectable levels in the case of some of the *Kudoa thrysites* counts. The feed attractants were then applied to the test diets at 2% of the total feed weight (wet basis) in the anchovy oil top coating. At this point, the *Kudoa* spores were either absent or present at an undetectable level. Therefore, there is the possibility that a dilution factor and the minimal amount consumed by the fish caused the level of *Kudoa* spores to be absent or undetectable.

Throughout the feeding trial, the water flow rate of the tanks was continuous. Due to the *Kudoa* spores being present in the anchovy oil top coating at the surface of the feed, there is the possibility that some of the parasites were washed away into the water and down the drain, prior to being consumed by the juvenile Chinook salmon. The time it took the fish to stop spitting out the feed pellets and to actually accept the feed may have also contributed to the washing out of the parasites into the water.

The juvenile Chinook salmon were also tested for the presence of *Kudoa* spore infections on day 0 and day 35 of the palatability feeding trial. There were no detectable levels of *Kudoa* parasites. Studies have documented that although some reared salmon species in British Columbia, like

Atlantic and Coho salmon, are very susceptible to *Kudoa* thrysites, Chinook salmon were not affected by these parasites (Whipps and Kent, 2006). Coho salmon have been shown to be highly susceptible to these parasites in net pens, while farmed Chinook salmon were resistant to the infections (Kent, 2000).

The absence of *Kudoa* paniformis in the Chinook salmon muscle tissue could have also been due to the species being resistant to these types of parasites. Another possibility is that the dilution factor through the feed, which was similar to the effect found with the fecal matter, rendered the levels to be undetectable. There are many methods that could be used to detect the *Kudoa* spores, such as PCR assays, microscopic examination, gross examination, and immunological techniques (Moran *et al*., 1999). Thus, methods like gross examination were also possible ways of detecting the presence of the parasites, since some studies have shown strong negative correlations between infection intensity and the firmness of the muscle tissue (Dawson-Coates *et al*., 2003). However, other researchers have found that infection threshold levels of around 20,000 spores per gram of muscle tissue must first be reached in order to see a physical difference in the fish muscle texture (St-Hilaire *et al*., 1997). This has been found with *Kudoa thrysites* in Atlantic salmon and *Kudoa paniformis* in Pacific hake (St-Hilaire *et al*., 1997). Another study showed that it takes approximately 5 to 6 months before the spores actually deteriorate the fish muscle tissue and before they can be detected (Moran *et al*., 1999). Therefore, it is possible that it was still too early to detect the spores in the salmon tissue. Although low levels of spores were present in the Pacific hake fish protein hydrolysates, additional filtering steps can be applied to the production process to completely remove the spores.

7.0 CONCLUSION

The use of Alcalase and Flavorzyme commercial enzymes and 1 and 4 hour hydrolysis times on Pacific hake created fish protein hydrolysates with different peptide and amino acid profiles. The Flavorzyme hydrolysates contained greater levels of free amino acids than the Alcalase hydrolysates. The 4 hour hydrolysates contained higher levels of free amino acids than the 1 hour hydrolysates. One of the Pacific hake hydrolysates used in the feeding trial proved to be an effective feed attractant, while the second hydrolysate did not have any significant impacts as a feed attractant. Hence, confirmation of the first null hypothesis (H_{o1}) depended upon what type of Pacific hake hydrolysate was used as the feed attractant. This was also the case with the second null hypothesis (H_{o2}), where one of the commercial feed attractants was an effective feed attractant, but the other did not show any significant results. The third null hypothesis (H_{o3}) was found to be true because there were no detectable signs of *Kudoa* spore transfer from the feed to the juvenile Chinook salmon.

Several of the feed attractants investigated in this study proved effective in increasing the palatability of the soybean meal diet. This was most pronounced in the 4 hour Alcalase hydrolysate, the commercial krill meal, and the commercial screen 1 attractant. With the exception of percent feed wasted, the fishmeal positive control diet was significantly superior to all of the soybean meal diets in all other palatability parameters. However, several of the feed attractants significantly improved feed intake, feed efficiency ratio, specific growth rate, size gain, and decreased percent feed wasted when compared with the soybean meal negative control diet, without a feed attractant.

This study provided evidence that feed attractants, in the form of hydrolyzed proteins, can act as effective palatability feed enhancer for Chinook salmon when applied to the feed as a top coating. Pacific hake, an inexpensive product, has the potential to be converted to a high quality feed attractant to help minimize the problems associated with soybean meal use in salmon aquaculture. This research helps address the need for finding an alternative use for Pacific hake, which are currently undervalued for their poor fillet texture caused by *Kudoa* spore infections.

Successful inclusion of a feed attractant into salmonid diets can benefit the salmon aquaculture industry in several ways. For example, it can decrease the expenses of running fish farms by reducing the amount of expensive fishmeal ingredients required. The feed attractants increase growth rates and market size of fish, giving rise to a more efficient aquaculture system. Use of plant proteins in the fish diets in place of fish protein may help decrease the environmental footprint of the feed used for Chinook salmon.

Future studies should focus on the development of effective salmon feed attractants. The feed attractants employed in this study were applied to the diets at 2% concentrations. Other studies could investigate dose response feeding trials and evaluate different application levels to see if these approaches would alter palatability. However, proficient growth in salmon requires a sufficient supply of all amino acids at the tissue sites of protein synthesis (Sveier *et* al., 2001b). Free amino acids are absorbed at a faster rate than protein bound amino acids and will enter the plasma pool much more efficiently (Sveier *et* al., 2001b). Therefore, the use of too high a concentration of fish protein hydrolysates may incorporate too high a concentration of certain free amino acids in the diet leading to an imbalance of free amino acids, peptides, and proteins in the digestion and absorption process of the fish (Espe *et al*., 1999). Soybean meal was included in the diets at 20%, which is higher than the average industrial application, especially for

juvenile salmon feeds. This was done intentionally, to look at the extreme side of an unpalatable diet. Therefore, further studies could reduce the soybean meal to more practical dietary concentrations to assess the effectiveness of the feed attractants. Finally, there were only five types of feed attractants used in this study. Future studies could investigate feed attractants produced with different proteases, hydrolysis parameters, and different marine-based starting materials.

8.0 TABLES AND FIGURES

Table 4.1. Production parameters for the four different types of whole Pacific hake fish protein hydrolysates (ALC1, ALC4, FLA1, FLA4).

FPH treatment[1]	**Hydrolysis temperature**	**Hydrolysis pH (initial-final)[2]**	**Hydrolysis time**	**Commercial enzyme (3%)[3]**
ALC1	50ºC	(7.21-6.42)	1 hour	Alcalase 2.4L
ALC4	50ºC	(7.21-6.33)	4 hours	Alcalase 2.4L
FLA1	50ºC	(7.23- 6.67)	1 hour	Flavorzyme 500L
FLA4	50ºC	(7.21-6.52)	4 hours	Flavorzyme 500L

[1]The 4 treatments: Alcalase 1 hour hydrolysis (ALC1), Alcalase 4 hour hydrolysis (ALC4), Flavorzyme 1 hour hydrolysis (FLA1), and Flavorzyme 4 hour hydrolysis (FLA4)
[2]Inherent pH was measured at time zero of the hydrolysis process and at the end of the hydrolysis process (time 1 hour or 4 hours).
[3]Commercial enzymes added at 3% of the total protein used to make the hydrolysate.

Table 4.2. Composition of Nutra Fry diet obtained from Skretting Canada Ltd., Vancouver, BC and fed to the juvenile Chinook salmon during the growth period.

Nutra fry 1.2 MM salmonid diet Guaranteed analysis (% as wet weight basis) [1]	
Crude protein (min)	50%
Crude fibre (max)	1.5%
Crude fat (min)	20%
Calcium (actual)	2.1%
Phosphorus (actual)	1.5%
Sodium (actual)	0.5%
Ash (max)	12.0%
Vitamin A (min)	5000 I.U.
Vitamin D_3 (min)	3000 I.U.
Vitamin E (min)	200 I.U.
Ingredients: Fishmeal, corn gluten meal, wheat flour, fish oil, poultry meal, feather meal, poultry fat, brewers yeast, A vitamin premix (vitamin A, vitamin D_3, vitamin E, inositol, calcium d-pantothenate, riboflavin, nicotinic acid, thiamine mononitrate, pyridoxine hydrochloride B_6, vitamin B_{12}, D-biotin, folic acid, ascorbyl polyphosphate C, and vitamin K), A mineral premix (manganese sulphate, zinc methionine, calcium iodate, copper sulphate, ferrous sulphate, sodium selenite, and betaine), and ethoxyquin (antioxidant).	

[1] Data acquired from the Skretting Canada Nutra Fry diet label.

Table 4.3. Composition of steam pelleted diet for juvenile Chinook salmon (formulated to contain 50% protein and 20% lipid on a dry weight basis)

Ingredients	Fish meal positive control diet in grams on a dry weight basis (dwb)[1]	Soybean meal test diets in grams on a dry weight basis (dwb)[2]
LT-anchovy meal; stabilized	516.7	389.8
Soybean meal	0.0	200.0[3]
Corn gluten meal	100.0	100.0
Wheat flour	157.9	47.7
Vitamin supplement[4]	20.0	20.0
Mineral supplement[5]	40.0	40.0
Anchovy oil; stabilized	136.1	151.3
Choline chloride (60%)	5.0	5.0
Vitamin C monophosphate (35%)	4.3	4.3
Permapell	20.0	20.0
DL-methionine	0.0	1.8
Ethoxyquin (Santoquin)	0.022	0.037
α-cellulose (negative control) or palatability enhancer (test diet)	0.0	20.0

[1] The fishmeal positive control diet with fishmeal (diet +CON from Table 4.4).
[2] The remaining diets contained soybean meal with different feed attractant coatings (diet –CON without a feed attractant coating, KRL with the 2% krill coating, ALC4 with the 4 hour Pacific hake hydrolysate coating, FLA4 with the 4 hour Pacific hake hydrolysate coating, SC1 with the EWOS screen 1 coating, and SC2 with the EWOS screen 2 coating from Table 4.4).
[3] Soybean meal was included at 20% of the total diet and 20.0% of the total protein in the diet.
[4] Vitamin supplement provided the following amounts/kg of diet (dwb). D-calcium panthothenate, 168.0 mg; pyridoxine HCl, 49.3 mg; riboflavin 60 mg; folic acid, 15 mg; thiamine mononitrate, 56 mg; biotin, 1.5 mg; cyanocobalmin (B_{12}), 0.09 mg; menadione (as MSBC), 18 mg; vitamin E, 300 IU; vitamin D_3, 2400 IU; vitamin A, 5000 IU; inositol, 400 mg; niacin, 300 mg; BHT, 22.0 mg. Wheat Starch was used as the carrier.
[5] Mineral supplement (mg/kg dwb): Fishmeal control diet; manganese (as $MnSO_4{\bullet}H_2O$), 65.6; zinc (as $ZnSO_4{\bullet}7H_2O$), 22.2; cobalt (as $COCl_2{\bullet}6H_2O$), 3; copper (as $CuSO_4{\bullet}5H_2O$), 5.8; iron (as $FeSO_4{\bullet}7H_2O$), 215; iodine (as KIO_3 and KI, 1:1), 10; fluoride (as NaF), 5; sodium (as NaCl), 846; selenium (as Na_2SeO_3), 0.15; magnesium (as $MgSO_4{\bullet}7H_2O$), 400. Soybean meal-based diet; Ca (as $CaHPO_4$), 2489; P (as $CaHPO_4$) 1923; manganese (as $MnSO_4{\bullet}H_2O$), 62.5; zinc (as $ZnSO_4{\bullet}7H_2O$), 40.4; cobalt (as $COCl_2{\bullet}6H_2O$), 3; copper (as $CuSO_4{\bullet}5H_2O$), 4.4; iron (as $FeSO_4{\bullet}7H_2O$), 184; iodine (as KIO_3 and KI, 1:1), 10; fluoride (as NaF), 5; sodium (as NaCl), 1967; selenium (as Na_2SeO_3), 0.15; magnesium (as $Mg\ SO_4{\bullet}7H_2O$), 138.

Table 4.4 Composition of oil coatings and ingredients used as protein sources in the 7 diets for the palatability feeding trial. The 7 diets included the positive control (+CON), the negative control (-CON), the krill meal (KRL), the 4 hour Alcalase (ALC4), the 4 hour Flavorzyme (FLA4), the screen 1 (SC1), and the screen 2 (SC2).

Diet[1]	Oil coating[2]	Base diet protein sources[4]	Type
+CON	Anchovy oil	Fish (51.67%), flour (15.79%)	Positive control
-CON	2% α-cellulose[3] in anchovy Oil	Fish (38.98%), flour (4.77%), soy (20%)	Negative control
KRL	2% krill meal in anchovy Oil	Fish (38.98%), flour (4.77%), soy (20%)	Test diet
ALC4	2% ALC4 in anchovy Oil	Fish (38.98%), flour (4.77%), soy (20%)	Test diet
FLA4	2% FLA4 in anchovy Oil	Fish (38.98%), flour (4.77%), soy (20%)	Test diet
SC1	2% screen 1 in anchovy Oil	Fish (38.98%), flour (4.77%), soy (20%)	Test diet
SC2	2% screen 2 in anchovy Oil	Fish (38.98%), flour (4.77%), soy (20%)	Test diet

[1] The 3 commercial attractants used were a spray dried krill meal powder from EWOS (KRL), an EWOS screen 1 test feed attractant (SC1), and an EWOS screen 2 test feed attractant (SC2). Please refer to Table 4.1 for ALC4 and FLA4 details.
[2] Anchovy oil was included at 13.61% of the total diet weight for the fishmeal positive control diet and 15.13% of the total diet weight for the remainder of the diets. The feed attractants were applied at 2% of the total diet weight, by mixing each of these into the supplemental anchovy oil and flour, followed by applying each as a top coating to the pelleted feed.
[3] α-Cellulose is a non-nutritive bulk material with no effect on fish palatability that was added to maintain consistency across the test diets (Higgs *et al*., 2006).
[4] Ingredients in the diet that contributed varying amounts of protein to the dietary protein content are shown as a percentage of the total diet dry weight. Low temperature (LT) anchovy meal ("fish"), wheat flour ("flour"), and soybean meal ("soy") are included; corn gluten meal also contributed to the total protein of the diet, but the protein source was not shown because it was included at the same level (10.00%) across all seven diets.

Table 4.5. Distribution of juvenile Chinook salmon into various tanks located at the Centre for Aquaculture and Environmental Research (CAER) during the growth period, acclimation period, feeding trial, and fecal collection period.

Date and Time	Tanks	Chinook salmon
July 26th, 2006 (growth period)	Spius Creek Hatchery Open water tank (1x)	5 g fish (3000 fish)
July 28th, 2006 (growth period)	CAER 1100L indoor tanks (3x)	5 g fish (1000 fish per tank)
July 29th, 2006 (growth period)	CAER 1100L indoor tanks (4x)	5 g fish (750 fish per tank)
August 30th, 2006 (acclimation period)	CAER 250L digestibility tanks (21x)	7-12 g fish (80 fish per tank)
	CAER 4000L outdoor tank (1x)	1320 fish
September 13th, 2006 (feeding trial)	CAER 250L digestibility tanks (21x)	7-12 g fish (75 fish per tank)
	CAER 4000L outdoor tank (1x)	1320 fish
October 20th, 2006 (fecal collection period)	CAER 250L digestibility tanks (21x)	7-15 g fish (70 fish per tank)
	CAER 4000L outdoor tank (1x)	1320 fish
October 28th , 2006	CAER 4000L outdoor tank (1x)	3000 fish (total does not account for sampling & fish mortality)

Table 4.6. Fish health checklist (Higgs *et al*., 2006). Guidelines for monitoring the health and well-being of the juvenile Chinook salmon throughout the growth period, acclimation period, feeding trial, and fecal collection stage of the experiment.

Intensity	Behavior	Color	Signs of disease
A	Swimming normally	Normal	Skin normal
B	Swimming at the top of the tank only	Slightly dark on back	Small red marks on base of fins or vent
C	Leaning against the side of the tank	Dark back and eyes	Large diffuse red areas
D	Disoriented (swimming upside down)		Open wounds or swelling

Table 5.1. Recovery of the whole Pacific hake fish protein hydrolysates (dry weight basis).

Fish protein hydrolysate[1]	Recovery[2] (% DB of whole fish)
ALC1	62.2 ± 0.6
ALC4	62.0 ± 0.1
FLA1	53.3 ± 1.0
FLA4	56.4 ± 1.8

[1] Please refer to Table 4.1 for full details on abbreviations.
[2] Values shown are the mean ± standard deviation results from quantification performed in triplicates.

Table 5.2. Proximate composition of the hydrolysates and commercial feed attractants.

Feed Coating[1]	% Crude Protein	% Fat	% Moisture	% Ash
	(Wet Weight Basis)[2]			
ALC1	81.4 ± 1.1	0.795 ± 0.330	3.48 ± 0.49	7.16 ± 0.57
ALC4	80.7 ± 0.2	0.144 ± 0.154	4.58 ± 1.22	6.14 ± 0.24
FLA1	75.4 ± 0.3	0.249 ± 0.103	4.82 ± 0.22	7.04 ± 0.23
FLA4	76.2 ± 0.9	0.140 ± 0.163	4.39 ± 0.24	6.39 ± 0.10
KRL	59.0 ± 0.1	18.2 ± 0.6	6.75 ± 0.01	11.5 ± 0.1
SC1	64.0 ± 0.4	10.7 ± 0.1	5.70 ± 0.06	22.9 ± 0.1
SC2	47.2 ± 0.6	9.23 ± 0.08	24.1 ± 0.1	19.7 ± 0.1

[1] Please refer to Table 4.1 and Table 4.4 for details on abbreviations.
[2] Values shown are the mean ± standard deviation results from quantification performed in triplicates.

Table 5.3. Total amino acid contents of the fish protein hydrolysates and commercial feed attractants [1] (g/100 g of sample). Values listed on a dry weight basis.

Amino acid	ALC1 Total	ALC4 Total	FLA1 Total	FLA4 Total	KRL Total	SC1 Total	SC2 Total
Asx (Asp+Asn)	5.32	5.28	4.87	5.5	6.62	4.42	3.61
Glx (Glu+Gln)	8.31	8.28	8.14	8.44	9.06	8.03	6.86
Ser	2.23	2.20	2.02	2.13	2.54	1.85	1.50
Gly	3.13	3.16	3.04	3.05	2.48	5.86	5.45
His	1.01	0.95	0.61	0.60	1.74	4.95	4.22
Tau	0.35	0.34	0.42	0.36	0.11	3.95	3.99
Arg	3.47	3.51	3.23	3.42	4.74	3.62	3.18
Thr	2.30	2.30	1.99	2.15	2.73	1.63	1.27
Ala	3.29	3.29	3.11	3.19	3.43	3.86	3.17
Pro	2.18	2.16	1.95	2.05	2.18	3.04	2.68
Tyr	1.58	1.58	1.23	1.42	3.15	0.85	0.57
Val	2.47	2.54	2.36	2.52	3.49	1.93	1.39
Met	1.64	1.66	1.44	1.55	2.15	1.23	1.01
Cys	1.37	1.34	1.42	1.36	0.08	0.02	0.01
Ile	2.12	2.11	1.97	2.14	3.46	1.23	0.88
Leu	4.05	4.05	3.71	3.87	5.22	2.25	2.28
Phe	1.96	1.94	1.58	1.74	2.57	0.95	0.69
Trp	0.13	0.17	0.19	0.23	0.00	0.08	0.07
Lys	4.65	4.15	3.94	4.03	3.70	2.24	2.09
Total	51.56	51.01	47.22	49.75	59.45	51.99	44.92

[1] Please refer to Table 4.1 and Table 4.4 for details about the abbreviations.

Table 5.4. Free amino acid contents of the fish protein hydrolysates and commercial feed attractants[1] (g/100 g of sample). Values listed on a dry weight basis.

Amino acid	ALC1 Free	ALC4 Free	FLA1 Free	FLA4 Free	KRL Free	SC1 Free	SC2 Free
Asp	0.20	0.30	1.24	1.03	n/a[2]	0.13	0.14
Glu	0.43	0.57	1.39	1.50	0.00	0.53	0.40
Asn	0.19	0.23	0.28	0.71	n/a[2]	0.01	0.02
Ser	0.41	0.43	0.97	1.13	0.00	0.12	0.18
Gln	0.38	0.39	0.86	0.81	0.00	0.18	0.15
Gly	0.13	0.11	0.44	0.52	0.05	0.30	0.24
His	0.11	0.11	0.10	0.14	0.24	4.95	4.22
Tau	0.32	0.27	0.36	0.33	0.10	3.95	3.99
Arg	0.79	0.99	1.76	1.98	0.13	0.28	0.29
Thr	0.38	0.44	0.93	1.08	0.00	0.31	0.23
Ala	0.73	0.73	1.39	1.62	0.02	1.06	0.82
Pro	0.08	0.06	0.09	0.10	0.09	0.30	0.27
Tyr	0.71	0.82	1.01	1.25	0.01	0.24	0.21
Val	0.49	0.57	1.37	1.58	0.01	0.45	0.32
Met	0.65	0.76	1.05	1.17	0.00	0.25	0.18
Cys	1.23	1.11	1.25	1.16	0.02	0.01	0.01
Ile	0.30	0.34	1.13	1.33	0.02	0.29	0.20
Leu	1.32	1.47	2.70	2.96	0.00	0.60	0.41
Phe	0.78	0.90	1.20	1.37	0.00	0.32	0.23
Trp	0.13	0.17	0.19	0.23	0.00	0.08	0.07
Lys	0.74	0.87	2.07	2.32	0.00	0.40	0.39
Total	10.5	11.64	21.78	24.32	0.69	14.16	12.97

[1] Please refer to Table 4.1 and Table 4.4 for details about the abbreviations.
[2] n/a = not analyzed.

Table 5.5. Free amino acid expressed as a percentage of total amino acid content of the fish protein hydrolysates and commercial feed attractants.[1]

Amino acid	ALC1 (%)	ALC4 (%)	FLA1 (%)	FLA4 (%)	KRL (%)	SC1 (%)	SC2 (%)
Asx (Asp+Asn)	7.3	10.0	31.2	31.6	0.0	3.2	4.4
Glx (Glu+Gln)	9.8	11.6	27.6	27.4	0.0	8.8	8.0
Ser	18.2	19.7	47.9	53.0	0.1	6.7	11.7
Gly	4.2	3.4	14.4	17.2	1.9	5.1	4.3
His	11.1	11.1	17.3	24.2	13.9	100.0	100.0
Tau	91.0	81.0	86.9	90.3	85.5	100.0	100.0
Arg	22.8	28.2	54.4	58.0	2.7	7.9	9.0
Thr	16.6	19.3	46.6	50.4	0.1	19.2	18.1
Ala	22.4	22.2	44.8	50.7	0.5	27.5	25.8
Pro	3.6	2.9	4.6	5.0	4.3	10.0	10.0
Tyr	44.7	52.2	82.0	87.8	0.2	28.4	37.2
Val	20.0	22.3	58.1	62.8	0.3	23.1	22.8
Met	39.9	45.7	72.9	75.3	0.2	20.6	17.8
Cys	89.7	83.0	87.9	85.4	23.0	40.5	69.6
Ile	14.0	16.3	57.4	62.3	0.6	23.6	23.2
Leu	32.5	36.4	72.8	76.6	0.1	0.0	18.0
Phe	39.6	46.2	76.0	78.6	0.2	33.7	34.0
Lys	16.0	21.0	52.5	57.6	0.1	17.6	18.7

[1] Please refer to Table 4.1 and Table 4.4 for details about the abbreviations.

Table 5.6. Saponin concentrations in the steam pelleted positive and negative control feeds. Values listed as wet weight basis.

Saponin	Fish meal positive control diet feed pellet (mg/g)	Soybean meal negative control diet feed pellet (mg/g)
Smilagenin	n/d[1]	n/d
Sarsasapogenin	n/d	n/d
Diosgenin	n/d	n/d
Tigogenin	n/d	n/d
Yamogenin	n/d	n/d
Neotigogenin/Beta-sitosterol	0.12	0.18
Yuccagenin	n/d	n/d
Gitogenin	n/d	n/d
Neogitogenin	n/d	n/d

[1] n/d = not detected (<0.05 mg/g), below the limit of detection for the method of analysis.

Table 5.7. Trace mineral levels and concentrations in the different diets.[1] Values listed as wet weight basis.

Metal	+CON (µg/g)	-CON (µg/g)	KRL (µg/g)	ALC4 (µg/g)	FLA4 (µg/g)	SC1 (µg/g)	SC2 (µg/g)
Aluminum	18	67.0	63.3	66.5	64.5	69.0	71.7
Antimony	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Arsenic	2.6	2.3	2.5	2.4	2.5	2.5	2.6
Barium	1.62	1.86	1.86	1.74	1.74	1.78	1.81
Beryllium	<0.01	<0.02	<0.01	<0.01	<0.01	<0.01	<0.01
Bismuth	<0.50	<0.50	<0.5	<0.5	<0.50	<0.5	<0.5
Cadmium	0.1	0.08	0.08	0.07	0.08	0.1	0.1
Calcium	11200	10700	10900	10600	10400	10700	10800
Chromium	0.514	0.600	0.543	0.564	0.596	0.533	0.792
Cobalt	2.6	1.0	0.74	5.31	1.3	3.7	1.5
Copper	8.18	7.08	8.39	7.06	7.30	11.9	7.14
Iron	370	267	262	285	240	231	222
Lead	0.4	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Lithium	1.5	1.4	1.4	1.4	1.4	1.4	1.4
Magnesium	1740	1780	1840	1770	1760	1830	1820
Manganese	95.2	58.2	49.8	53.2	49.3	61.4	69.3
Molybdenum	0.2	1.9	2.0	1.9	2.0	1.9	2.0
Nickel	0.53	2.2	2.1	2.2	2.2	2.0	2.2
Phosphorus	12600	12400	12500	12400	12400	12700	12500
Potassium	10900	13600	13600	13700	14000	14400	14100
Selenium	6.58	1.6	1.8	1.8	1.7	2.0	1.7
Silver	<0.1	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1
Sodium	6440	5510	6030	5910	5880	7030	6530
Strontium	13.4	14.0	19.6	13.8	13.7	14.2	14.5
Titanium	<0.05	4.4	4.2	4.4	4.2	4.4	4.7
Vanadium	<0.1	<0.2	<0.1	0.2	<0.1	<0.1	<0.1
Zinc	102	100	101	99.8	92.4	103	91.7
Zirconium	<0.05	0.4	0.4	0.3	0.3	0.4	0.4
Thallium	<0.2	2.2	2.3	2.0	1.9	2.5	2.4

[1] Please refer to Table 4.1 and Table 4.4 for details about the abbreviations.

Table 5.8. Spore counts of *Kudoa paniformis* and *thrysites* for whole Pacific hake fish mince (pooled from 41 fish) and Pacific hake fish protein hydrolysates produced from it. Quantification was done in triplicate.

Sample	*Kudoa* spore	Average infection intensity (spores/gram)	Standard error
Whole Fish	*K. paniformis*	5.29×10^6	3.83×10^5
	K. thyrsites	1.67×10^4	8.33×10^3
ALC1 (1 hour Alcalase)	*K. paniformis*	8.33×10^3	8.33×10^3
	K. thyrsites	8.33×10^3	4.17×10^3
ALC4 (4 hour Alcalase)	*K. paniformis*	1.67×10^4	1.10×10^4
	K. thyrsites	4.17×10^3	4.17×10^3
FLA1 (1 hour Flavorzyme)	*K. paniformis*	2.08×10^4	8.33×10^3
	K. thyrsites	n/d[1]	n/d
FLA4 (4 hour Flavorzyme)	*K. paniformis*	3.75×10^4	1.91×10^4
	K. thyrsites	4.17×10^3	4.17×10^3

[1] n/d = not detected (<6.25×10^3 spores/gram of sample), below the limit of detection for the method of analysis.

Figure 5.1. Size exclusion chromatography for the four Pacific hake hydrolysates (ALC1, ALC4, FLA1, FLA4) monitored by absorbance at 214nm. Arrows indicate elution of molecular weight markers at fraction 18 (14,300 Da), 33 (1355.38 Da), and 40 (226.24 Da).

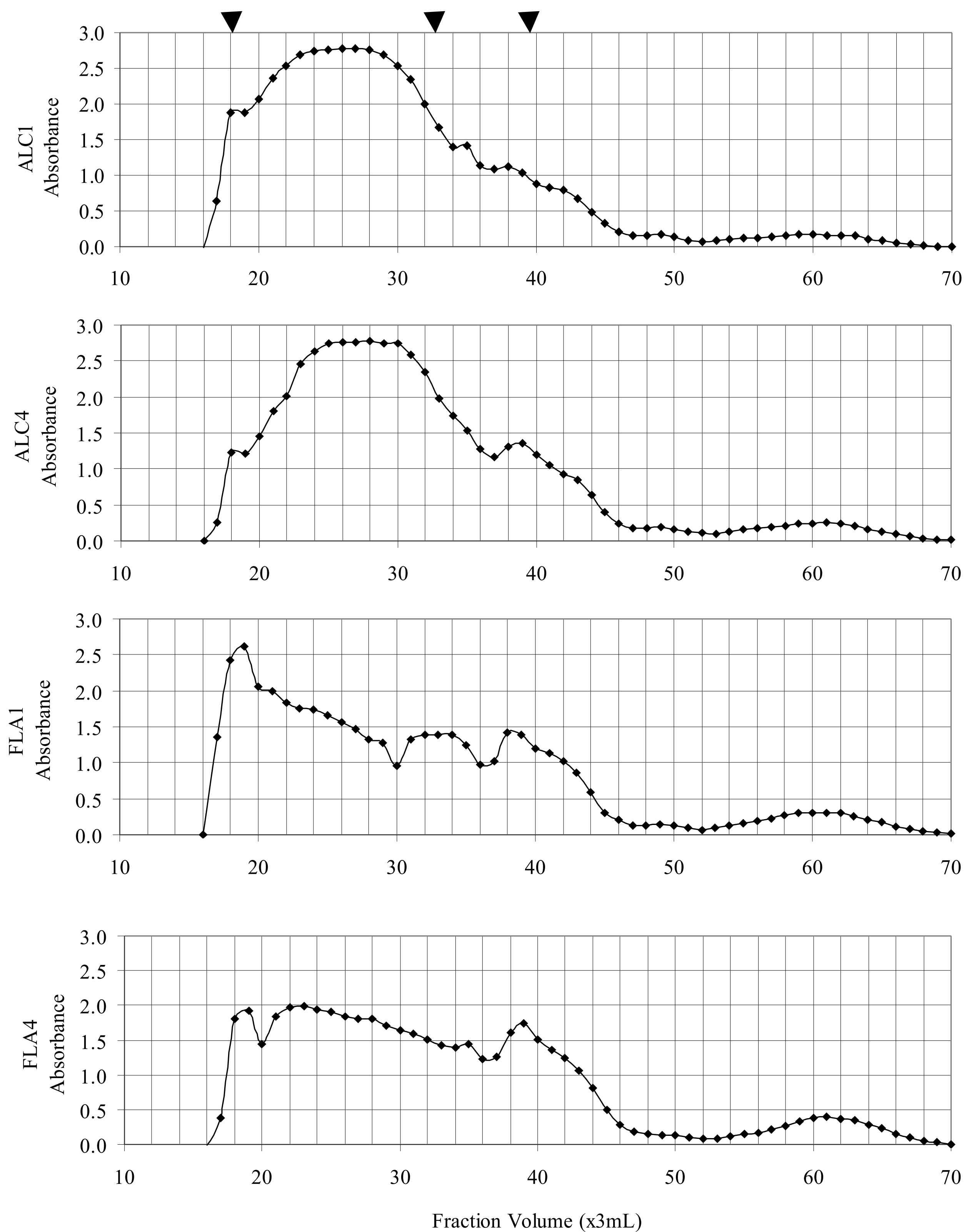


Figure 5.2. Size exclusion chromatography for the four Pacific hake hydrolysates (ALC1, ALC4, FLA1, FLA4) monitored by absorbance at 230nm. Arrows indicate elution of molecular weight markers at fraction 18 (14,300 Da), 33 (1355.38 Da), and 40 (226.24 Da).

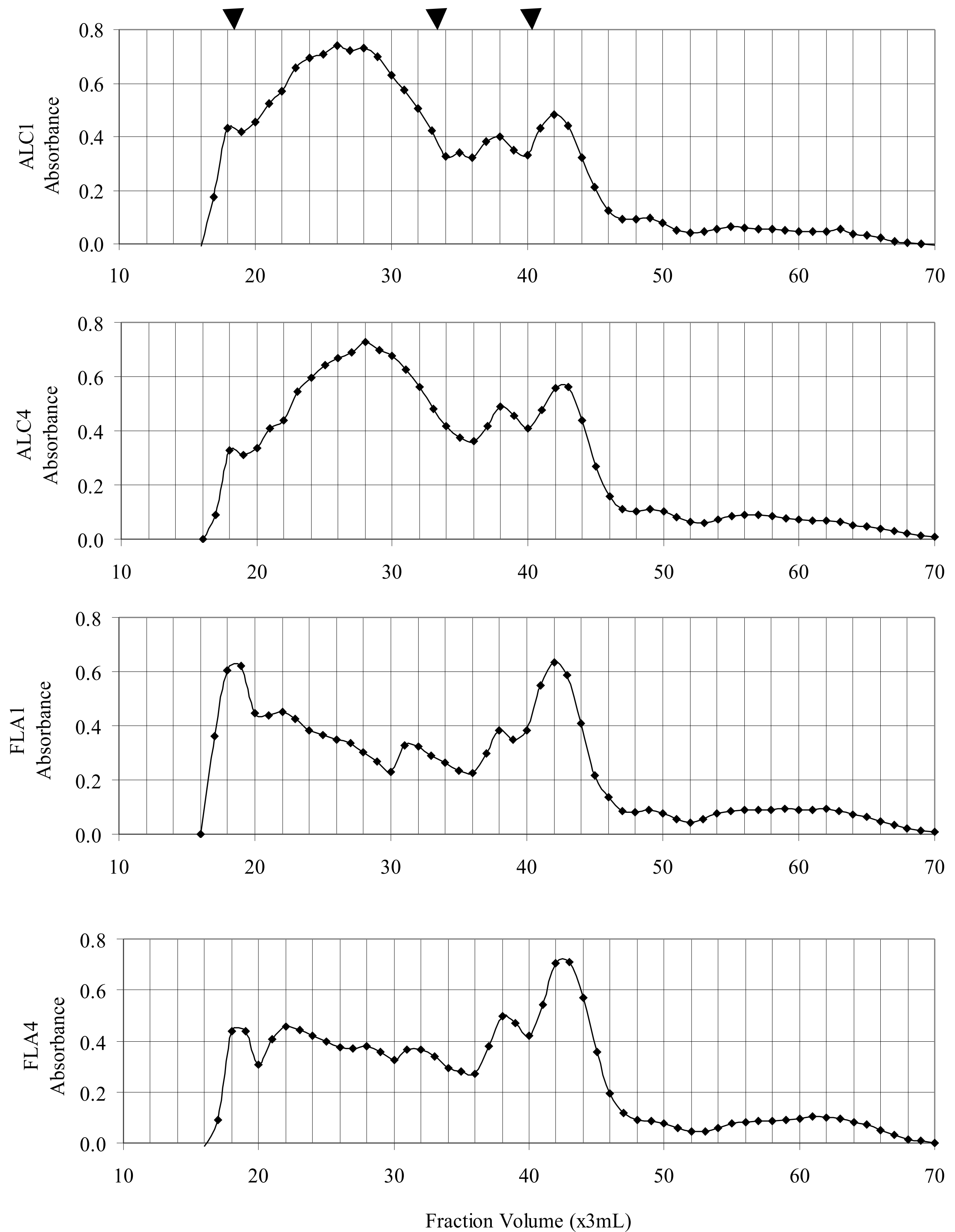


Figure 5.3. Size exclusion chromatography for the four Pacific hake hydrolysates (ALC1, ALC4, FLA1, FLA4) monitored by absorbance at 280nm. Arrows indicate elution of molecular weight markers at fraction 18 (14,300 Da), 33 (1355.38 Da), and 40 (226.24 Da).

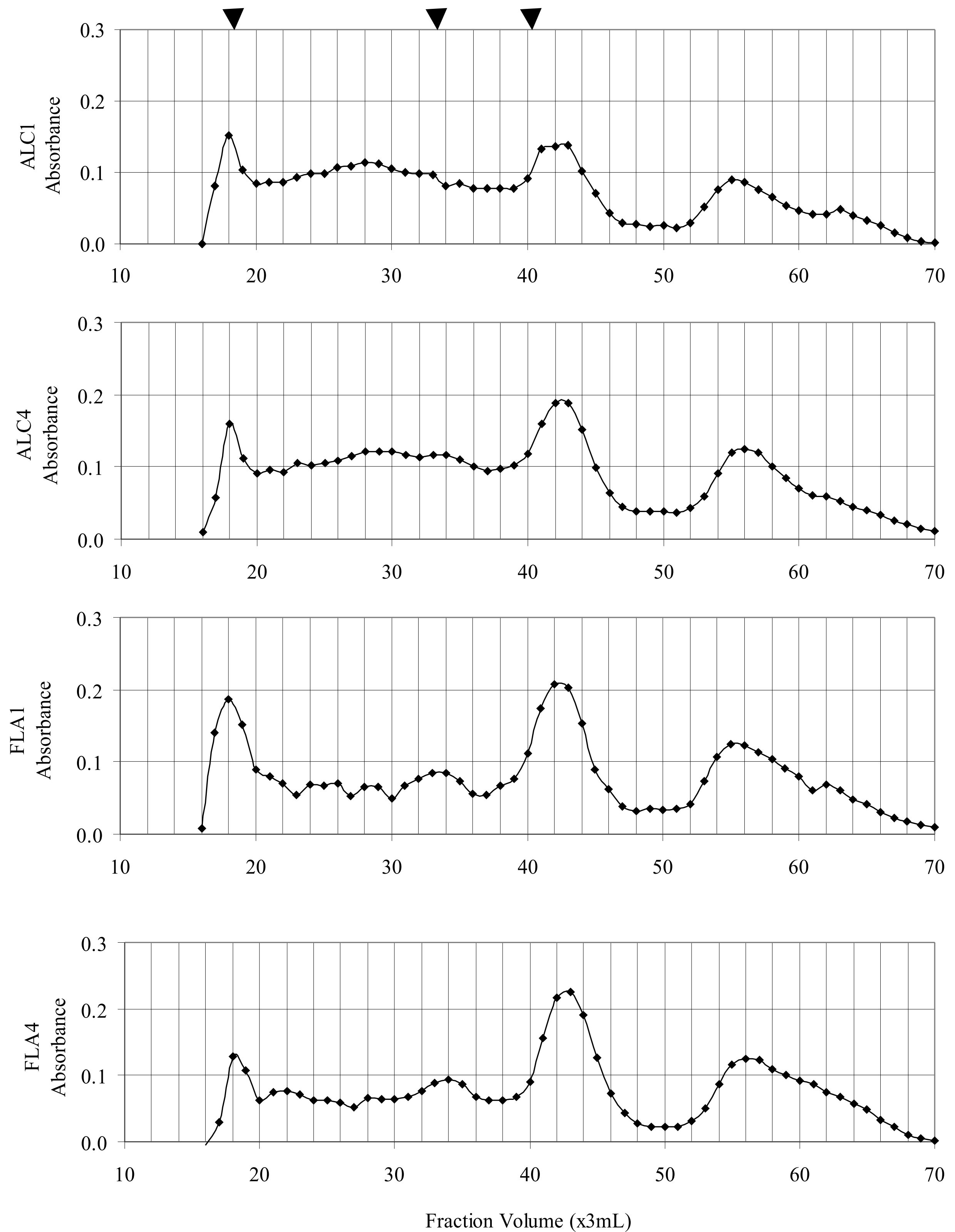


Figure 5.4. Size exclusion chromatography for the three commercial feed attractants (SC1, SC2, KRL) monitored by absorbance at 214nm. Arrows indicate elution of molecular weight markers at fraction 18 (14,300 Da), 33 (1355.38 Da), and 40 (226.24 Da).

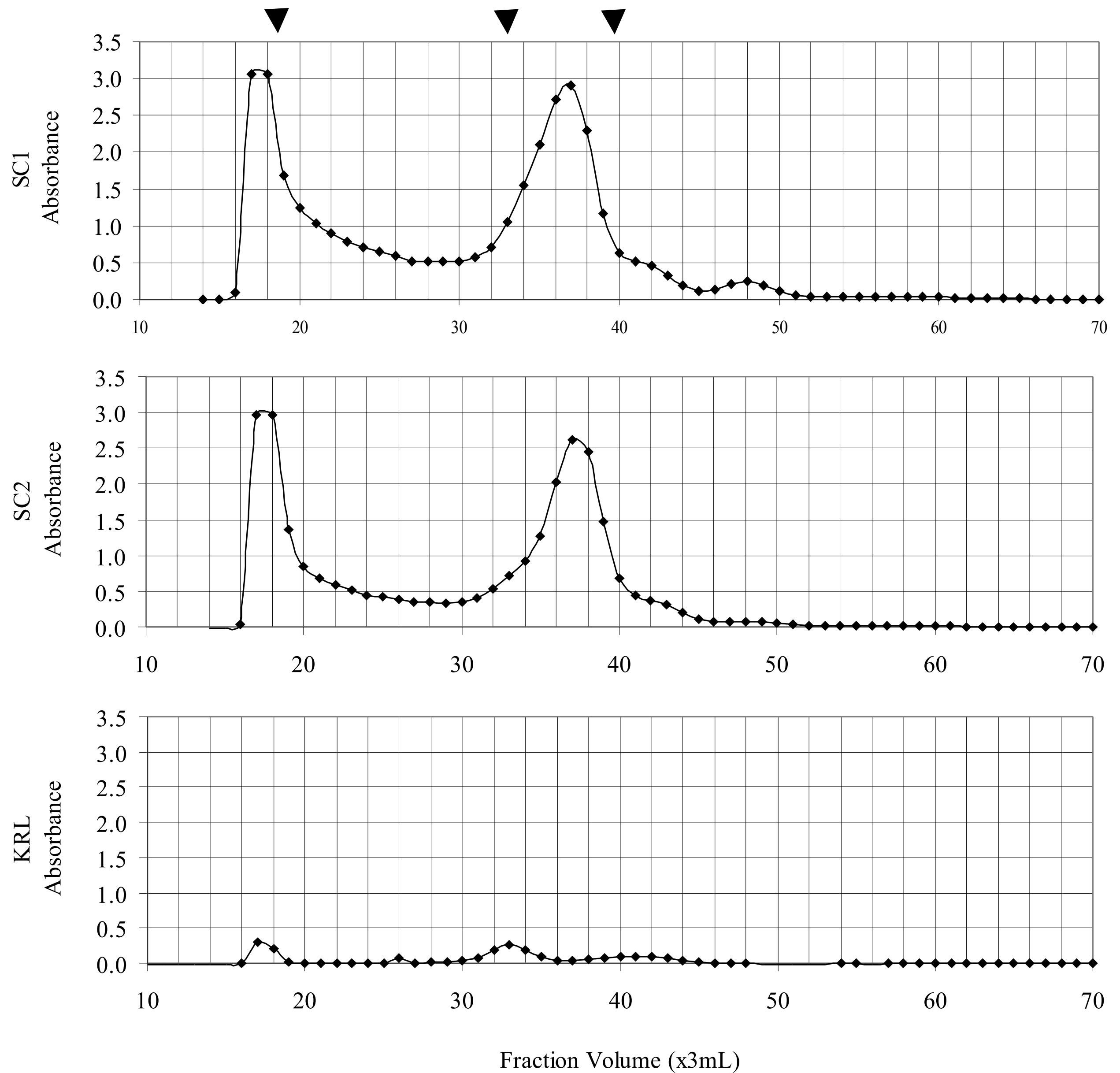


Figure 5.5. Size exclusion chromatography for the three commercial feed attractants (SC1, SC2, KRL) monitored by absorbance at 230nm. Arrows indicate elution of molecular weight markers at fraction 18 (14,300 Da), 33 (1355.38 Da), and 40 (226.24 Da).

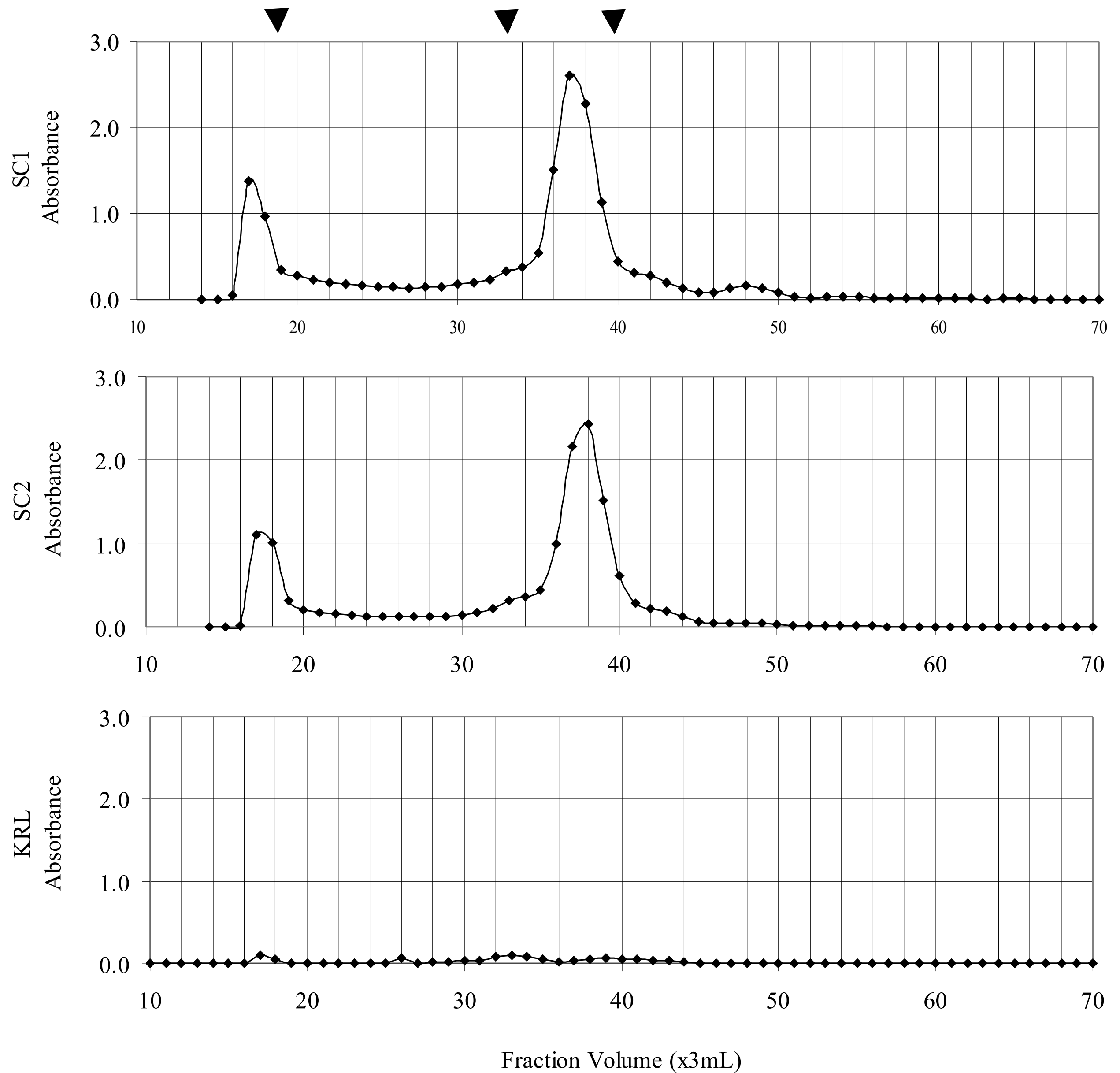


Figure 5.6. Size exclusion chromatography for the three commercial feed attractants (SC1, SC2, KRL) monitored by absorbance at 280nm. Arrows indicate elution of molecular weight markers at fraction 18 (14,300 Da), 33 (1355.38 Da), and 40 (226.24 Da).

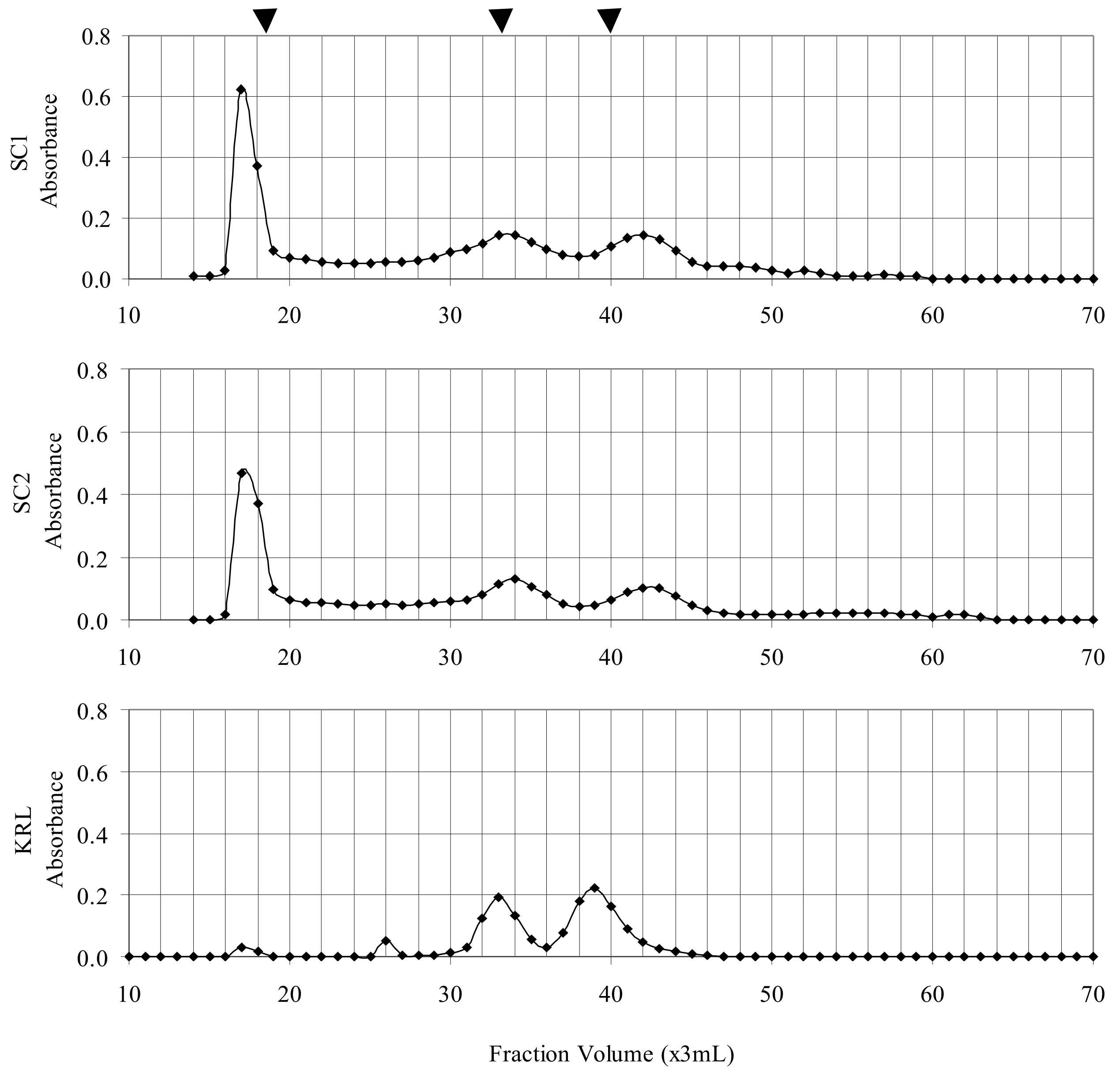


Figure 5.7. Mean (n=3) daily dry feed intake (± SEM) for juvenile Chinook salmon fed the different test diets (please refer to Table 4.1 and Table 4.4 for abbreviations). Means that do not share a common letter are significantly different ($p < 0.05$).

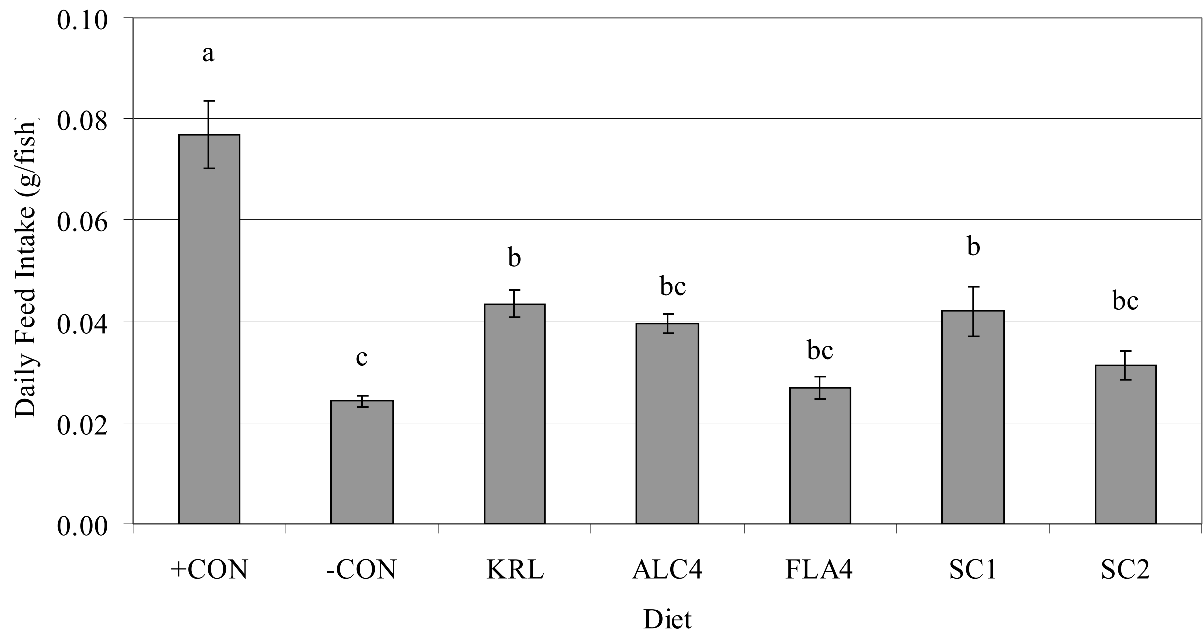


Figure 5.8. Mean (n=3) total dry feed intakes (± SEM) for juvenile Chinook salmon fed the different diets over the 35-day period (please refer to Table 4.1 and Table 4.4 for abbreviations). Means that do not share a common letter are significantly different ($p < 0.05$).

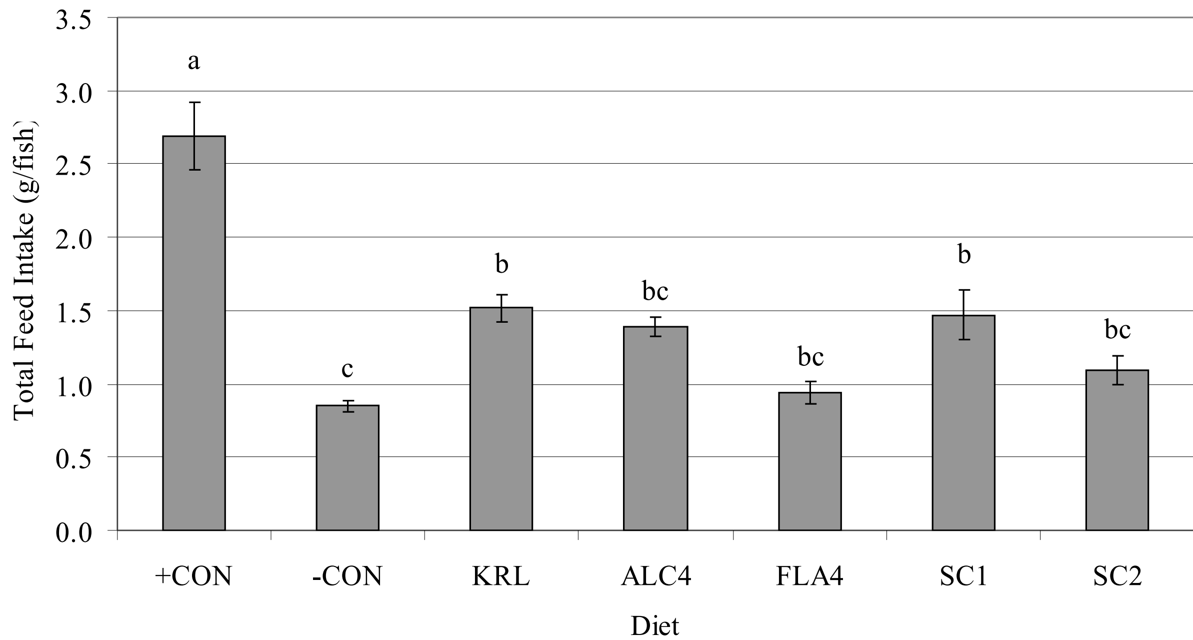


Figure 5.9. Mean (n=3) feed efficiency ratios (± SEM) for juvenile Chinook salmon on the different diet treatments (please refer to Table 4.1 and Table 4.4 for abbreviations). Feed efficiency ratio is the amount of body mass increase in the fish to the amount of dry feed consumed in grams. Means that do not share a common letter are significantly different ($p < 0.05$).

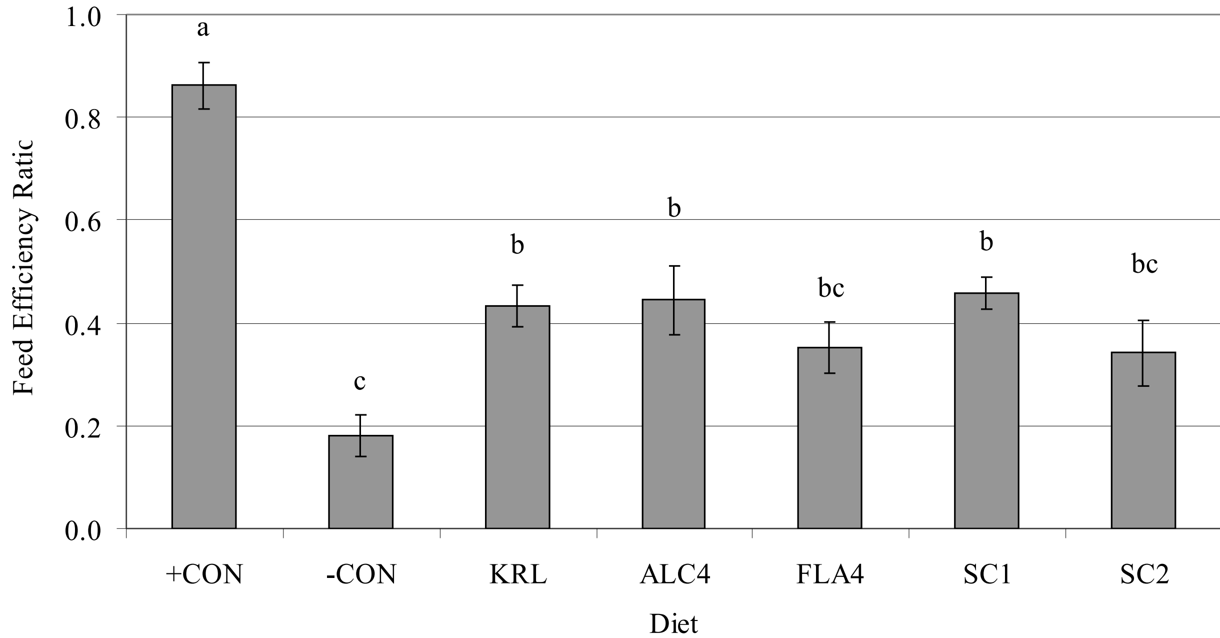


Figure 5.10. Mean (n=3) specific growth rates (± SEM) measured as percent increase of the salmon's body weight or length per day, for juvenile Chinook salmon fed the different test diets (please refer to Table 4.1 and Table 4.4 for abbreviations). Means within each parameter (weight and length) that do not share a common letter are significantly different ($p < 0.05$).

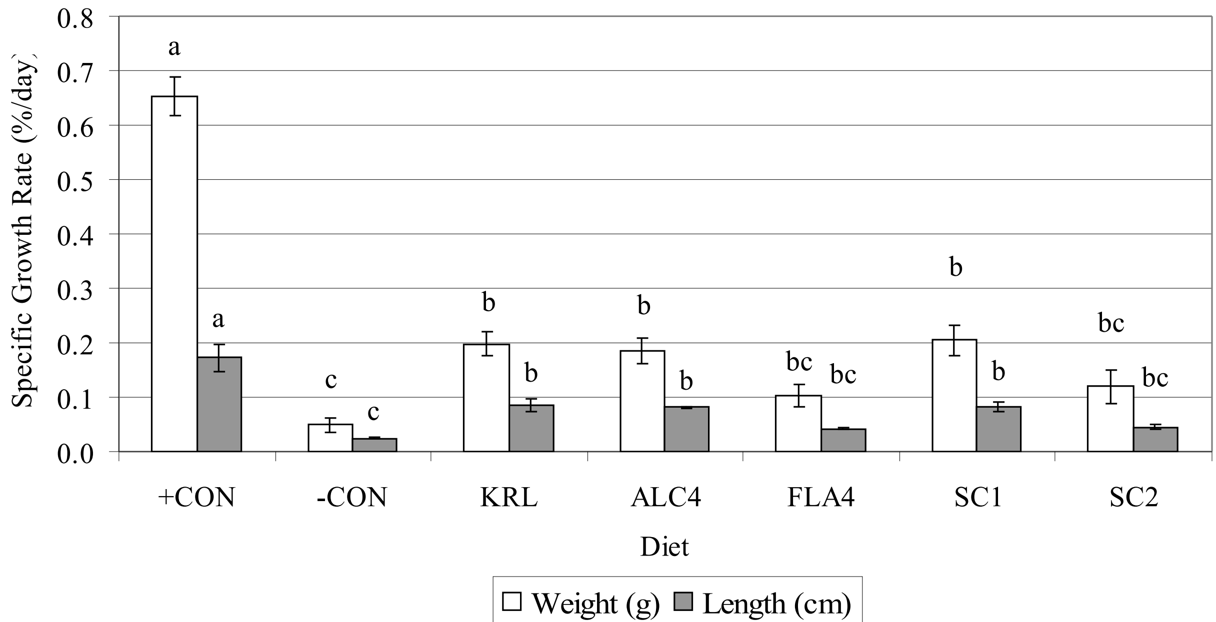


Figure 5.11. Average (n=3) weight (g) and length (cm) gains (± SEM) for juvenile Chinook salmon fed the different diets treatments (please refer to Table 4.1 and Table 4.4 for abbreviations). Test means within each parameter (weight and length) that do not share a common letter are significantly different ($p < 0.05$).

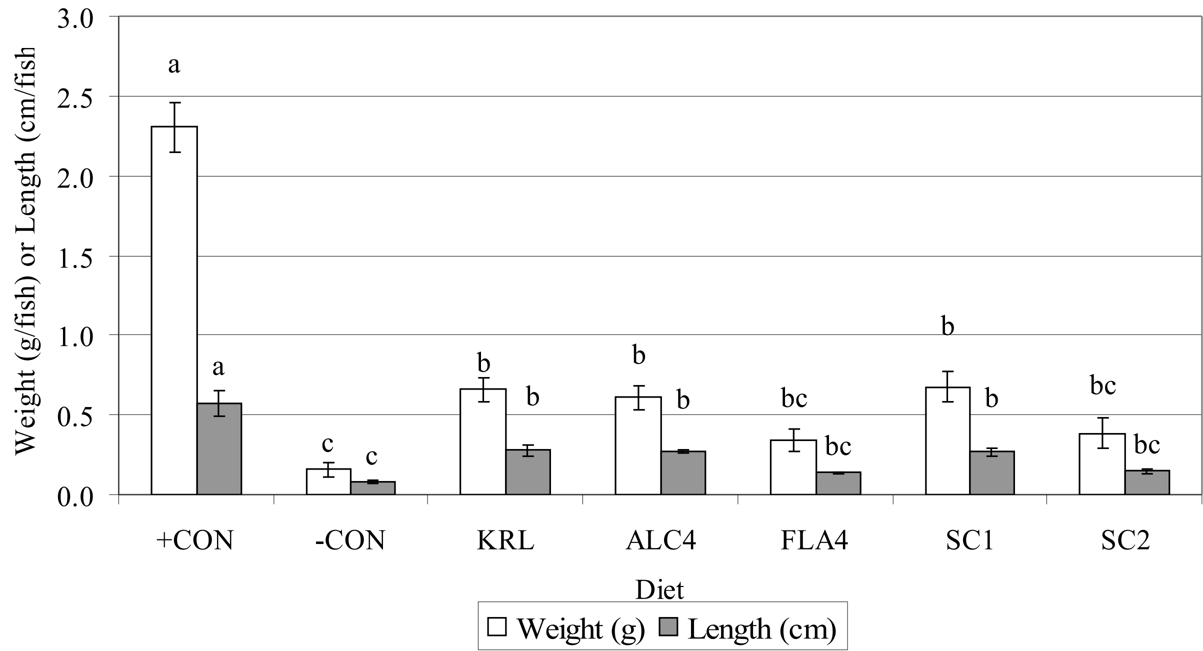


Figure 5.12. Mean (n=3) total amounts of feed dispensed in grams (± SEM) for juvenile Chinook salmon given each dietary treatment (please refer to Table 4.1 and Table 4.4 for abbreviations). The dark gray area represents the total amount of feed consumed by fish of that specific diet. White areas represent the total amount of feed that was not consumed and wasted by the fish. Diet treatments within each parameter (total feed wasted and total feed intake) that do not share a common letter are significantly different ($p < 0.05$).

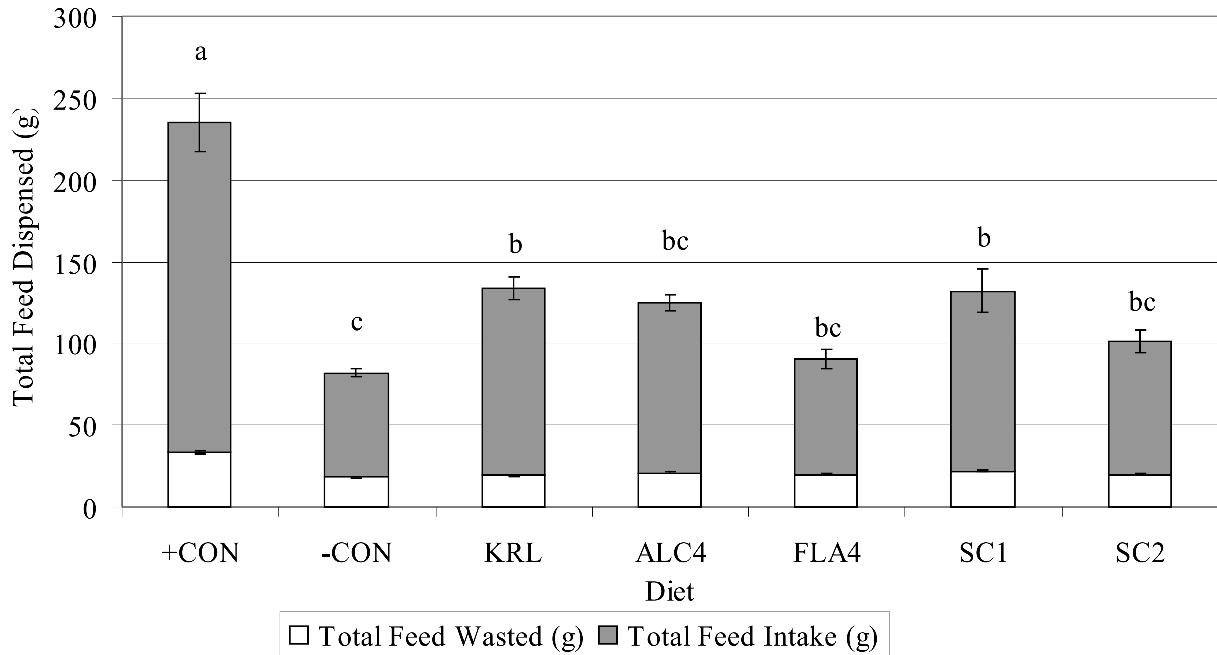


Figure 5.13. Mean (n=3) percentages of feed wasted (± SEM) of the feed dispensed for juvenile Chinook salmon fed the different diets (please refer to Table 4.1 and Table 4.4 for abbreviations). Means that do not share a common letter are significantly different ($p < 0.05$).

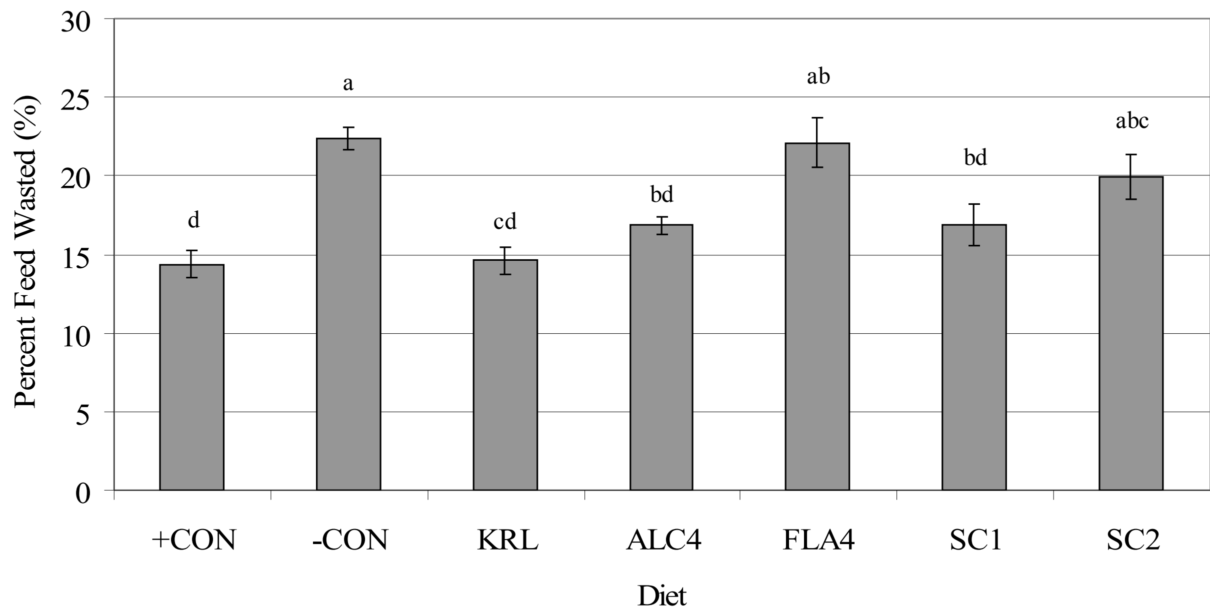
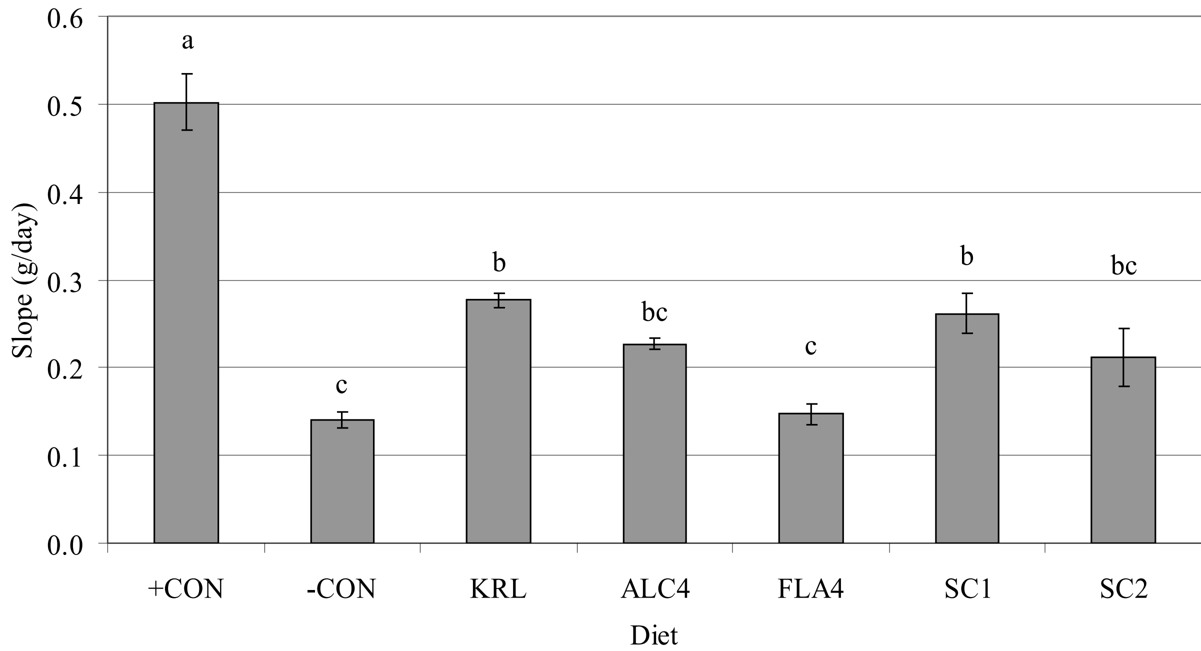


Figure 5.14. Acclimation trend for juvenile Chinook salmon given the different dietary treatments (please refer to Table 4.1 and Table 4.4 for abbreviations). Values shown are the means and standard errors for each diet (n=3), and diets that do not share a common letter are significantly different ($p < 0.05$).



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