PHYSIOLOGICAL MECHANISMS OF NUTRITIONAL ADAPTATION TO PLANT PROTEIN DIETS BY SELECTIVE BREEDING IN RAINBOW TROUT

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AUTHORIZATION TO SUBMIT DISSERTATION

This dissertation of Andreas Brezas, submitted for the degree of Doctor of Philosophy with a major in Animial Physiology and titled “PHYSIOLOGICAL MECHANISMS OF NUTRITIONAL ADAPTATION TO PLANT PROTEIN DIETS BY SELECTIVE BREEDING IN RAINBOW TROUT,” has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

A selected rainbow trout strain was used as a model to identify physiological parameters associated with improved performance when fed all plant-protein feed. Results from a digestibility trial showed that selection had no measurable effect on apparent digestibility coefficients of nutrients in the all plant-protein or a conventional fishmeal-based diet. While this result validated apparent digestibility as an evaluation tool for ingredient and diet quality, it demonstrated that other physiological mechanisms are responsible for the improved performance of the selected strain when fed an all plant-protein, high-soy diet. A subsequent experiment was conducted to evaluate the effects that alternate protein ingredients and plant protein mixtures with or without amino acid supplementation have on trout digestive physiology. Results from temporal plasma amino acids measurements at the absorption site (hepatic portal vein) and from the systemic blood (caudal vein) demonstrated that plasma amino acid level in the hepatic portal vein, measured at intervals following a single meal, are a valuable tool to evaluate the effects of candidate alternate proteins on fish digestive physiology. The findings showed that each ingredient affects digestive physiology of the fish in a singular manner when ingredients are fed individually. However, they do not have any predictable additive effect when fed together as a mixture. Moreover, the addition of crystalline amino acids to an all-plant protein mixture altered the plasma concentrations of all the amino acids as it did for uptake reflected in the hepatic portal vein. A major finding in the study was that the selected trout strain fed the plant protein mixture with amino acid supplementation showed a noteworthy difference compared to an unselected strain, specifically, a synchronous and homogenous decreasing pattern for all the essential amino acids over time in the hepatic portal vein. This indicates that homogeneous dietary amino acid uptake in the hepatic portal vein and rapid postprandial plasma amino acid disappearance are results of nutritional adaptation driven by selection for growth on and tolerance of all-plant protein diet. Results from gene expression of amino acid transporters, cholecystokinin and genes related with protein and amino acid metabolism supported the findings from the plasma amino acids. In conclusion, the

results of the research described in this dissertation demonstrated that improved performance of the selected trout strain is associated with synchronous protein digestion of the plant protein mixture and synchronization of amino acid absorption leading to improved amino acid availability and utilization.

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DEDICATION

This dissertation is dedicated to the memory of my beloved grandmother

Γιαννούλα Μπρέζα

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CHAPTER 1

INTRODUCTION

Aquaculture is the rearing of fish, shrimps, other crustaceans, shellfish and aquatic plants for consumption. Aquaculture history can be traced back almost 4000 years (Costa-Pierce 2010). In ancient times aquaculture rearing systems were similar to natural production systems. This type of aquaculture required minimal inputs and is termed extensive rearing; its equivalent in livestock production would be grazing. In modern times, aquaculture growth and expansion are based upon systems in which inputs, such as fry or juveniles and feed, are supplied in confined and managed conditions. This type of aquaculture is termed intensive, or if water quality is actively managed, super intensive (Verdegem et al., 2006). Extensive systems are characterized by a limited stocking density with limited or no external inputs. In practice fish are confined in a pond or lagoon and fish growth and overall fish production is based on nutrition from natural productivity with no external feed used. If some feed inputs are supplied but natural productivity still supplies a significant percentage of nutrition, it is termed a semi-intensive system. Intensive systems evolved to increase productivity of extensive or semi-intensive systems. First, the stocking density of good quality fingerlings is much higher extensive or semi-intensive system resulting in much higher final biomass produced per unit of surface area or rearing volume. High quality feed that meets the species and the life stage requirements for optimal growth is supplied and the water quality is monitored and managed. Finally, super intensive systems are the most advanced production system. The high biomass produced per unit of surface area or volume is made possible by total control of water quality and other conditions (e.g. temperature) in such a way that the production cycle is kept at a minimum.

Global aquaculture production (fish, shellfish, mollusks, crustaceans and plants) in 2016 reached 110.2 million tonnes, with the estimated production of finfish reported to be 54.1 million tonnes with an economic value of $138.5 billion USD (FAO, 2018). Aquaculture is a food producing industry that is expected to play a major role

in the future of the ‘’feeding of humanity’’. However, according to a United Nations Department of Economic and Social Affairs (UN-DESA) report published in 2015, the world population of 7.3 billion is expected to reach 8.5 billion by 2030 while projections for 2050 and 2100 are 9.7 and 11.2 billion, respectively (UN-DESA, 2015). Fish is considered a high-quality food and aquaculture has a carbon footprint lower than other animal production systems (Béné et al., 2015). From 1973 to 2000 the global consumption of fish as human food doubled in quantity (Delgado et al., 2003). The Food and Agriculture Organization of the United Nations (FAO) has reported that since 1961, the average annual increase in global fish consumption is 3.2 percent and in 2015 the global per capita consumption of fish and other aquatic products was 20.2 Kg (FAO, 2018). In order for aquaculture to continue playing this pivotal role as a major food producing sector and to satisfy increased human demands for protein, aquaculture needs to grow steadily and in a sustainable manner. Despite the fact that aquaculture growth rates in 1980s and 1990s were reported to be extremely high (10.8 and 9.5 percent, respectively), its global growth rate declined to a moderate 5.8 percent during the period 2001 to 2016 (FAO, 2018). However, aquaculture still is considered to be the fastest growing major food production sector.

Recently, major questions have arisen regarding the way that aquaculture should expand in terms sustainability. Currently, according to the FAO, even though marine aquaculture has been growing rapidly for the past decade, marine finfish production accounted for only 6.6 million tonnes out of 54.1 million tonnes in total and fish production was dominated by inland (freshwater) aquaculture (FAO, 2018). The big question is, if human population numbers will reach projected levels, will the planet’s carrying capacity in terms of fresh water resources, support the tremendous growth of aquaculture needed to meet future demand? From what is projected for freshwater resources, that will not be feasible. Therefore, aquaculture production growth will have to be based upon the marine ecosystem making the oceans a major source of food (Duarte, 2009).

Sustainable aquaculture production for fish, shrimp and crustaceans relies heavily on the use of feed. Most of the species that are currently produced worldwide

are farmed under intensive conditions requiring a considerable amount of feed to be supplied. In 2016, filter-feeding fish species (extensive system) production was reported to be only 13.6 million tonnes (~25% of total production), this clearly shows the need for feed as a necessary input for aquaculture production (FAO, 2018). Taking into consideration future production targets that aquaculture needs to reach, global fish feed production will have to likewise increase to provide the nutrients necessary for future fish under intensive culture to thrive and grow. Fish feed represents up to 60% of production costs, with dietary protein being the single most expensive component accounting for nearly half of the cost of aquafeed (NRC, 2011). Dietary proteins are essential for the normal growth of animals. Therefore, maximizing protein utilization by the animal is considered a very important factor to manage feed costs and improve sustainable production.

For many years ‘’standard’’ production diets for finfish were based on fishmeal as the primary protein source. Global fishmeal production for the past three decades varied from year to year but has remained steady and is unlikely to increase beyond current average amounts. In contrast the aquaculture sector continuously demands larger quantities of this commodity. While in the early 1990s and up until 2007 the average price of fish meal was around $500/metric ton (mt), it increased after that reaching $1200/mt in 2009, and in recent years fishmeal prices reached $1500/mt (Olsen and Hasan 2012). Finite supplies and increasing demand associated with increasing, intensive production of marine carnivorous fish and shrimp species are mainly responsible for increased fishmeal prices. In 1988, the reported percentage of global fishmeal production used by the aquaculture sector was 10%. By 2010 the percentage used by aquaculture was estimated to be around 56% (Olsen and Hasan 2012). Today, the percentage is estimated to be 75% (Tacon and Metian, 2008).

Fishmeal is considered to be a superior ingredient for many reasons, including its high protein content, excellent amino acid profile, high nutrient digestibility, adequate amounts of micronutrients as well as a general lack of anti-nutrients (Larsen et al., 2012). Fishmeal is a complicated matrix which contains large amounts of essential nutrients and also a considerable number of biologically active compounds.

Fishmeal is rich in macro and trace minerals which are also highly bioavailable such as phosphorus (Hardy 2010). Fishmeal in general terms is a product obtained by cooking, pressing, drying and grinding whole small pelagic fish or fish by-products from the fish processing industry. There are many different categories of fishmeal based upon its processing characteristics, source of the raw material and the chemical composition of the final product. Fishmeal features unique characteristics as an ingredient for fish nutrition. Being a product from fish, its amino acid profile closely resembles those of a majority of farmed fish species. Fishmeal has a fat content that varies from 7-11%, with a fatty acid composition unique among all other livestock feed ingredients. It has a high content of highly unsaturated, omega-3 fatty acids that are essential nutrients to fish, specifically Eicosapentaenoic *Acid* (EPA), Docosapentaenoic Acid (DPA) and *Docosahexaenoic Acid* (DHA). The omega-3 (n-3) content of fishmeal is considerably higher than omega-6 (n-6) polyunsaturated fatty acids commonly found in plant lipids. It is rich in phospholipids and supplies cholesterol to the diet which is necessary to fish. Fishmeal also provides other non-essential nutrients to the fish which have beneficial physiological actions such as hydroxyproline and taurine (Kousoulaki et al., 2009). Plant proteins lack these compounds. Finally, fishmeal appears to also contain unidentified growth factors (Hardy, 2010).

For the past three decades many efforts have been made to replace fishmeal in fish feeds with proteins of animal, plant or microbial origin (NRC, 2011). Land animal proteins, produced from byproducts of livestock and poultry processing, are effective and economical replacements but their use has been restricted at times by national and international regulations (NRC, 2011). Single cell proteins, also called microbial proteins, are high protein ingredients that have considerable potential as fish feed ingredients, but remain rather expensive (Ritala et al., 2017). Protein concentrates of plant origin are proposed as the main alternate protein sources to supply protein for aquafeeds because of their abundance and relative cost compare to fishmeal. Alternative plant protein sources currently being used are produced from grains, including corn, wheat, rice and barley, oilseeds including soybean, canola, rapeseed

and cottonseed, pulses, including peas and lupins, and tubers such as potatoes. The most commonly used products in fish feeds are soybean meal, soy protein concentrate, corn gluten meal, wheat gluten meal, rapeseed meal and sunflower meal (Hardy, 2010). All are produced from the residue remaining after oil extraction for human use.

During the past several decades a plethora of research has been conducted towards substituting fishmeal with plant proteins. However, numerous studies have shown suboptimal fish growth performance and reduced protein retention efficiency when fish are fed low fishmeal - high plant protein feeds (Gomes et al., 1995; Xie et al., 1997; Davies and Morris, 1997; Yamamoto et al., 2000; Refstie et al., 2000; Martin et al., 2003; De Francesco et al. 2004; Gomez-Requeni et al., 2004; Palmegiano et al., 2006; Panserat et al., 2008). Plant proteins have some characteristics that make their use challenging (Francis et al., 2001; Gatlin et al., 2007; Hardy, 2010). Plant protein amino acid profiles differ from animal amino acid profiles, do not match the dietary amino acid requirements of fish essential amino acids and typically provide insufficient amounts of lysine, methionine and threonine. Ultimately, leading to levels that are considered to be limiting in diets when formulation is based on the plant protein ingredients (Ahmed et al., 2019). Plant proteins also often contain compounds that are considered as antinutritional factors to fish. For example, non-starch polysaccharides are a negative component of plant ingredients because they are not digestible to fish and also do not provide energy from microbial fermentation in the intestine of carnivorous fish (Stone, 2003). They may also reduce nutrient utilization and thereby reduce feed efficiency, although a complete understanding of such antinutritional actions is lacking (Gatllin et al., 2007). Plant seeds possess mechanisms of defense to discourage their consumption. Some plant seeds contain protease inhibitors and lectins that can cause allergic reactions in fish. Storage proteins in soybean include glycinin and β-conglycinin that also induce nonspecific inflammatory reactions in fish (Rumsey et al., 1994). Lectins are well known to act as antinutritional factors. Lectins are actually glycoproteins which are also called agglutinins and they are present in plants. Lectins bind to fish intestinal epithelia

leading to pathological changes associated with nutrient absorption (Buttle et al., 2001). Phyto-estrogenic compounds in plants like daidgein, quercitin and genistein, are substances which negatively affect reproductive performance in fish (Gatlin et al., 2007). Another major antinutritional factor present in plants is phytate or phytic acid which is the main storage form of phosphorus in seeds. Phytic acid is associated with reduced digestibility of phosphorus, lower availability of zinc and reduced apparent digestibility of protein among other effects (Sugiura et al., 2001; Gatlin et al., 2007). Other plant products such as β-glucans are mainly found in the bran of seeds like barley, wheat, and oats. They have immunomodulatory effects which can be positive or negative depending upon the duration of the fish feeding period. Short-term intake might be beneficial (Fehringer et al., 2014). However, for the past several years fish feeds containing β-glucans have been used through almost the entire grow out period, without clear evidence of either negative or positive effects on fish performance. Glucosinolates are compounds which are not harmful to animals and fish in their native form but instead their hydrolysis products like goitrin and thiocyanate cause physiological alterations that affect the uptake of iodine by the thyroid (Burel et al., 2001). Gossypol is another compound of plant origin which is toxic to the fish especially in regards to reproduction (Lee and Dabrowski, 2002). Finally, alkaloids are a class of bitter tasting compounds that can affect feed palatability and also are toxic to fish (Halver and Hardy, 2002).

Even though plant derived feed ingredients contain antinutritional factors that limit high inclusion rates in aquafeeds, technologies are available which can inactivate antinutrients or reduce their effects. First of all, for heat-labile compounds like protease inhibitors and lectins, appropriate thermal processing, i.e., temperature and duration of exposure, can in large part inactivate them (Drew et al., 2007). Heat stable compounds like saponins, phytic acid, phytoestrogens, non-starch polysaccharides, glucosinolates and protein antigens can be removed by various processes such as dehulling, aqueous or solvent extraction, fractionation or with exogenous enzymes (Drew et al., 2007).

Evaluation of alternative proteins as potential ingredients for aquafeeds begins with chemical analysis of proximate constituents and essential nutrient levels, followed by assessment of digestibility. Digestibility is an indicator of the digestive process efficiency of either a nutrient or an ingredient and is usually expressed as a percentage (Moyano et al., 2015). Digestibility in fish nutritional science is based upon disappearance of nutrients in feeds and their content in feces. Measuring nutrient digestibility for ingredients or diets means measuring the amount of ingested nutrients and energy that is not excreted in feces (Glencross et al., 2007). Reliable data on nutrient digestibility are crucial in the evaluation of the potential inclusion of feed ingredients in diets to develop least-cost feed formulations and to minimize the environmental impact of animal production (Sales, 2009). Gaylord et al. (2008) developed a data set of apparent digestibility coefficients (ADC) using extruded diets for 24 ingredients commonly used in aquafeeds and several candidate ingredients. Among the ingredients were five fishmeals, three animal by-products, five plant protein concentrates and four high protein (>25%) plant meals. The results showed that fishmeals and the animal by-products had similar ADC protein values while the plant protein concentrates showed the highest values among the tested ingredients. In more detail ADC protein values for menhaden fishmeal was the lowest (86%), followed by Mexican sardine meal (89%), special select menhaden and regular sardine meal (90%) and 97% for anchovy fish meal. Animal by-product meals showed 87% for the feather meal, 88% for poultry by-product meal and 91% for spray-dried poultry blood meal. Plant protein concentrates also had high ADC protein values with soy protein concentrate (SPC) and wheat gluten meal (WGM) calculated at 99% and 100%, respectively. Corn gluten meal (CGM) and barley protein concentrate recorded 92%; the lowest ADC protein value of 89% was measured for rice protein concentrate. High protein oilseed meals gave lower values than plant protein concentrates, 89% for soybean meal and 75% for cottonseed meal, canola meal and flaxseed meal (Gaylord et al., 2008). The results of the above study confirmed results of other studies (Kaushik et al., 1995; Sugiura et al., 1998; Bureau et al., 1999; Cheng and Hardy 2002; Glencross et al., 2005).

However, digestibility is not something fixed but rather reflects an interaction between the ingredient or the feed and the animal which processes it (Fuller, 2012). Determination of the availability of dietary amino acids is a central concept in the study of nutrition. However, limited information exists on the apparent and true amino acid availability values from animal and plant protein sources (Lall and Anderson 2005). The term “availability” has been one that has caused considerable confusion sometimes with the term digestibility. Availability is a measure of the disappearance from the gut lumen of dietary amino acid during digestion, but more commonly has been used to describe the release, uptake and subsequent post-absorptive utilization of dietary amino acids. However, availability of an amino acid may be quite different from the empirically determined digestibility value or from the ultimate degree of utilization. The latter measure is dependent upon a number of dietary and animal factors and is highly variable (Moughan, 2003). The bioavailability of nutrients has been defined as the proportion of ingested nutrients from a particular source that is absorbed in a form that can be utilized during metabolism by animals (NRC, 2011). The bioavailability of nutrients is usually measured in the blood plasma (Moyano et al., 2018).

Protein quality describes various properties of a protein in relation to its ability to achieve defined metabolic actions. Traditionally, this has been discussed solely in the context of a protein’s ability to provide specific quantities of amino acids to satisfy the demands for synthesis of protein as measured by animal growth (Millward et al., 2008). Limited information exists for apparent and true amino acid availability values from animal and plant protein sources. Efforts to completely replace fishmeal with alternate protein sources in salmonids feeds has the risk of creating a deficiency or imbalance of dietary amino acids (Lall and Anderson 2005; Larsen et al., 2012). A distortion of amino acid physiological levels could contribute to the difficulties encountered when fish are fed diets containing alternative protein sources (Rolland et al., 2015). Protein synthesis in cells requires all essential amino acids to be available at the moment proteins are being made. If one essential amino acid is not present in sufficient amounts, remaining amino acids are quickly metabolized for energy (NRC,

2011). This results in lower protein retention efficiency and increased protein turnover, a common finding when fish are fed plant plant-based feeds (Davies and Morris, 1997; Refstie et al., 2000; Martin et al., 2003; Ambardekar et al., 2009). Asynchronous amino acid intestinal absorption may also alter the activity of mTOR (mechanistic or mammalian target of rapamycin), the master regulator of skeletal muscle protein turnover in cells (Zargar et al., 2011; Weichhart, 2012). Post-prandial changes of plasma amino acids levels have been the subject of investigation of many research papers (Schuhmacher et al., 1997; Yamamoto et al., 1988; Ok et al., 2001; Karlsson et al., 2006; Larsen et al., 2012; Yun et al., 2016). What is well known is the fact that crystalline amino acid supplementation to a diet alters the pattern of temporal bioavailability of amino acids, with the crystalline amino acids being absorbed and appearing in the plasma much faster than amino acids originated from intact proteins (Yamada et al., 1981; Ambardekar et al., 2009). Feeding a diet in which the amino acid profile is deficient in one or more amino acids will limit protein deposition, limit the retention of the other amino acids, and force their deamination and catabolism (NRC, 2011). However very few research projects have focused on evaluating single ingredients (Yamada et al., 1981; Murai et al., 1987; Ambardekar et al., 2009) or have compared diets which based their formulations on practical ingredients (Yamamoto et al., 1988; Schumacher et al.,1997; Karlsson et al., 2006; Larsen et al., 2012). Measuring post-prandial free amino acid profiles could reveal valuable information about the factors that contribute to growth in fish when fishmeal is replaced by alternate protein sources. Furthermore, this approach could provide insights regarding the digestive physiology of certain fish species or strains in terms of their response to feed formulation changes. Another fact that should be considered is that most of the studies conducted to measure plasma amino acids analyze blood taken from the dorsal aorta which makes it difficult to extrapolate amino acid absorption rate from the intestine and/or the hepatic metabolism. Only a few studies have taken this factor into consideration and have sampled blood from the hepatic portal vein (Murai et al., 1987; Karlsson et al., 2006; Ambardekar et al., 2009). Even though many studies have analyzed plasma amino acid concentrations and their post-prandial patterns in fish after feeding single ingredients and purified, semi-purified or practical diets, these

studies did not result in major advancements towards fishmeal replacement. The main reason for this discrepancy is the fact that the desired amino acid post-prandial pattern of absorption and the desired post-prandial pattern of plasma amino acids in peripheral tissues are not known. It is known that the plasma amino acid patterns observed in fish after feeding fishmeal-based diets are very different than patterns measured when fish are fed plant protein-based diets (Yamamoto et al., 1988; Larsen et al., 2012). However, the fact that fishmeal is the only single ingredient that a diet can be formulated with in contrast to plant protein blends contributes to the problem.

Six generations of selective breeding of rainbow trout for growth performance traits when fed high-soy feeds has resulted in a selected trout line (UI/USDA). This strain grows rapidly and efficiently when fed all plant-protein feeds containing 45% soy products, unlike unselected trout that exhibited 10-15% lower growth and feed efficiencies than the selected strain (Overturf et al., 2013). The selected rainbow trout strain is also a unique model to identify genetic and physiological parameters associated with soy protein utilization in fish. Animal models have been used to address a variety of scientific questions, from basic science to the development and assessment of novel vaccines, or therapies. Nutritional studies with animals, poultry and fish commonly involve supplementing negative control diets with essential nutrients or feed ingredients and measuring growth or other response variables. Response variables in treatment groups are compared to those measured in animals in the negative control group. Experiments are sometimes designed to contain positive control groups, i.e., treatment groups where the conditions of the experiment are expected to guarantee a positive result. The selected trout strain grows twice as rapidly as parental lines and does so when fed a high-soy, all plant-protein feed (Overturf et al., 2013). Furthermore, the selected strain has been shown to be less sensitive to plant origin antinutritional factors and does not develop intestinal inflammation (Venold et al., 2012). These traits make it possible for the selected strain to be considered a “positive-control” to compare with non-selected trout strains. This approach presents the possibility to correlate positive responses with differences in physiological measurements that should provide novel insights into physiological

mechanisms responsible for the high performance exhibited by the select strain when fed a high-soy, all plant-protein feed.

The research described in this dissertation was undertaken to test the hypothesis that increased protein turnover and reduced muscle protein accretion associated with feeding high soy plant protein-based diets are the result of an asynchronous protein digestion, uptake and delivery of amino acids causing differential expression of genes controlling protein degradation pathways. The results of the research may yield new knowledge that can be used to formulate plant-based feeds to avoid this effect on protein turnover. These developments may also provide a solid scientific basis for improving the sustainability of aquaculture and increasing soy protein use in feeds for a range of farmed fish.

1.1 Bibliography

Ahmed, M., H. Liang, H. C. Kasiya, K. Ji, X. Ge, M. Ren, B. Liu, X. Zhu, and A. Sun. 2019. “Complete Replacement of Fish Meal by Plant Protein Ingredients with Dietary Essential Amino Acids Supplementation for Juvenile Blunt Snout Bream (*Megalobrama amblycephala*).” *Aquaculture Nutrition* 25(1):205–14.

Ambardekar, A. A., R. C. Reigh, and M. B. Williams. 2009. “Absorption of Amino Acids from Intact Dietary Proteins and Purified Amino Acid Supplements Follows Different Time-Courses in Channel Catfish (*Ictalurus punctatus*).” *Aquaculture* 291(3–4):179–87.

Béné, C., M. Barange, R. Subasinghe, P. Pinstrup-Andersen, G. Merino, G. Hemre, and M. Williams. 2015. “Feeding 9 Billion by 2050 – Putting Fish Back on the Menu.” *Food Security* 7(2):261–74.

Borey, M., S. Panserat, A. Surget, M. Cluzeaud, E. Plagnes-Juan, A. Herman, V. Lazzarotto, G. Corraze, F. Médale, B. Lauga, and C. Burel. 2016. “Postprandial Kinetics of Gene Expression of Proteins Involved in the Digestive Process in

Rainbow Trout (*O. mykiss*) and Impact of Diet Composition." *Fish Physiology and Biochemistry* 42(4):1187–1202.

Bureau, D., A. Harris, and C. Cho. 1999. "Apparent Digestibility of Rendered Animal Protein Ingredients for Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture* 180(3–4):345–58.

Burel, C., T. Boujard, S. J. Kaushik, G. Boeuf, K. A. Mol, S. Van der Geyten, V. M. Darras, E. R. Kühn, B. Pradet-Balade, B. Quérat, A. Quinsac, M. Krouti, and D. Ribaillier. 2001. "Effects of Rapeseed Meal-Glucosinolates on Thyroid Metabolism and Feed Utilization in Rainbow Trout." *General and Comparative Endocrinology* 124(3):343–58.

Buttle, L. G., A. C. Burrells, J. E. Good, P. D. Williams, P. J. Southgate, and C. Burrells. 2001. "The Binding of Soybean Agglutinin (SBA) to the Intestinal Epithelium of Atlantic Salmon, *Salmo Salar* and *Rainbow Trout*, *Oncorhynchus mykiss*, Fed High Levels of Soybean Meal." *Veterinary Immunology and Immunopathology* 80(3–4):237–44.

Cheng, Z. J. and R. W. Hardy. 2002. "Effect of Microbial Phytase on Apparent Nutrient Digestibility of Barley, Canola Meal, Wheat and Wheat Middlings, Measured in Vivo Using Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture Nutrition* 8(4):271–77.

Costa-Pierce, B. A. 2010. "Sustainable Ecological Aquaculture Systems: The Need for a New Social Contract for Aquaculture Development." *Marine Technology Society Journal* 44(3):88–112.

Davies, S. J. and P. C. Morris. 1997. "Influence of Multiple Amino Acid Supplementation on the Performance of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), Fed Soya Based Diets." *Aquaculture Research* 28(1):65–74.

Delgado, C. L., N. Wada, M. W. Rosegrant, S. Meijer, and M. Ahmed. 2003. “Fish to 2020: Supply and Demand in Changing Global Markets.”

Drew, M. D., T. L. Borgeson, and D. L. Thiessen. 2007. “A Review of Processing of Feed Ingredients to Enhance Diet Digestibility in Finfish.” *Animal Feed Science and Technology* 138(2):118–36.

Duarte, C. M., M. Holmer, Y.Olsen, D. Soto, N. Marbà, J. Guiu, K. Black, and I. Karakassis. 2009. “Will the Oceans Help Feed Humanity?” *BioScience* 59(11):967–76.

Fehringer, T. R., R. W. Hardy, and K. D. Cain. 2014. “Dietary Inclusion of Salmon Testes Meal from Alaskan Seafood Processing Byproducts: Effects on Growth and Immune Function of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum).” *Aquaculture* 433:34–39.

de Francesco, M., G. Parisi, F. Médale, P. Lupi, S. J. Kaushik, and B. M. Poli. 2004. “Effect of Long-Term Feeding with a Plant Protein Mixture Based Diet on Growth and Body/Fillet Quality Traits of Large Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 236(1–4):413–29.

Francis, G., H. P. S. Makkar, and K. Becker. 2001. *Antinutritional Factors Present in Plant-Derived Alternate Fish Feed Ingredients and Their Effects in Fish*. Vol. 199.

Fuller, M. 2012. “Determination of Protein and Amino Acid Digestibility in Foods Including Implications of Gut Microbial Amino Acid Synthesis.” *British Journal of Nutrition* 108(S2):S238–46.

Gatlin, D. M., F. T. Barrows, P. Brown, K. Dabrowski, T. G. Gaylord, R. W. Hardy, E. Herman, G. Hu, Å. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E. J. Souza, D. Stone, R. Wilson, and E. Wurtele. 2007. “Expanding the Utilization of Sustainable Plant Products in Aquafeeds: A Review.” *Aquaculture Research* 38(6):551–79.

Gaylord, T. G., F. T. Barrows, and S. D. Rawles. 2008. “Apparent Digestibility of Gross Nutrients from Feedstuffs in Extruded Feeds for Rainbow Trout, *Oncorhynchus mykiss*.” *Journal of the World Aquaculture Society* 39(6):827–34.

Glencross, B. D., M. Booth, and G. L. Allan. 2007. “A Feed Is Only as Good as Its Ingredients - A Review of Ingredient Evaluation Strategies for Aquaculture Feeds.” *Aquaculture Nutrition* 13(1):17–34.

Glencross, B., D. Evans, K. Dods, P. McCafferty, W. Hawkins, R. Maas, and S. Sipsas. 2005. “Evaluation of the Digestible Value of Lupin and Soybean Protein Concentrates and Isolates When Fed to Rainbow Trout, *Oncorhynchus mykiss*, Using Either Stripping or Settlement Faecal Collection Methods.” *Aquaculture* 245(1–4):211–20.

Gomes, E. F., P. Rema, and S. J. Kaushik. 1995. “Replacement of Fish Meal by Plant Proteins in the Diet of Rainbow Trout (*Oncorhynchus mykiss*): Digestibility and Growth Performance.” *Aquaculture* 130(2–3):177–86.

Gómez-Requeni, P., M. Mingarro, J. a. Calduch-Giner, F. Médale, S. a M. Martin, D. F. Houlihan, S. Kaushik, and J. Pérez-Sánchez. 2004. “Protein Growth Performance, Amino Acid Utilisation and Somatotropic Axis Responsiveness to Fish Meal Replacement by Plant Protein Sources in Gilthead Sea Bream (*Sparus aurata*).” *Aquaculture* 232(1–4):493–510.

Hardy, R. W. 2010. “Utilization of Plant Proteins in Fish Diets: Effects of Global Demand and Supplies of Fishmeal.” *Aquaculture Research* 41(5):770–76.

Karlsson, A., E. J. Eliason, L. T. Mydland, A. P. Farrell, and A. Kiessling. 2006. “Postprandial Changes in Plasma Free Amino Acid Levels Obtained Simultaneously from the Hepatic Portal Vein and the Dorsal Aorta in Rainbow Trout (*Oncorhynchus mykiss*).” *Journal of Experimental Biology* 209(24):4885–94.

Kaushik, S. J., J. P. Cravedi, J. P. Lalles, J. Sumpter, B. Fauconneau, and M. Laroche. 1995. “Partial or Total Replacement of Fish Meal by Soybean Protein on Growth, Protein Utilization, Potential Estrogenic or Antigenic Effects, Cholesterolemia and Flesh Quality in Rainbow Trout, *Oncorhynchus mykiss*.” *Aquaculture* 133(3–4):257–74.

Kousoulaki, K., S. Albrektsen, E. Langmyhr, H. J. Olsen, P. Campbell, and A. Aksnes. 2009. “The Water Soluble Fraction in Fish Meal (Stickwater) Stimulates Growth in Atlantic Salmon (*Salmo salar L.*) given High Plant Protein Diets.” *Aquaculture* 289(1–2):74–83.

Lall, S. P. and Anderson, S. 2005. “Amino Acid Nutrition of Salmonids: Dietary Requirements and Bioavailablity.” *Cahiers Options Méditerranéennes* 63(May):1–90.

Larsen, B. K., J. Dalsgaard, and P. B. Pedersen. 2012. “Effects of Plant Proteins on Postprandial, Free Plasma Amino Acid Concentrations in Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 326–329:90–98.

Lee, K. J. and K. Dabrowski. 2002. “Gossypol and Gossypolone Enantiomers in Tissues of Rainbow Trout Fed Low and High Levels of Dietary Cottonseed Meal.” *Journal of Agricultural and Food Chemistry* 50(10):3056–61.

Martin, S. A. M., O. Vilhelmsson, F. Médale, P. Watt, S. Kaushik, and D. F. Houlihan. 2003. “Proteomic Sensitivity to Dietary Manipulations in Rainbow Trout.” *Biochimica et Biophysica Acta* 1651(1–2):17–29.

Millward, D. J., D. K. Layman, D. Tomé, and G. Schaafsma. 2008. “Protein Quality Assessment: Impact of Expanding Understanding of Protein and Amino Acid Needs for Optimal Health.” *The American Journal of Clinical Nutrition* 87(5):1576S–1581S.

Moughan, P. J. 2003. “Amino Acid Availability: Aspects of Chemical Analysis and Bioassay Methodology.” *Nutrition Research Reviews* 16(02):127.

Moyano, F. J., M. A. Saénz de Rodrigáñez, M. Díaz, and A. G. J. Tacon. 2015. “Application of *in Vitro* Digestibility Methods in Aquaculture: Constraints and Perspectives.” *Reviews in Aquaculture* 7(4):223–42.

Ok, I. H., S. C. Bai, G. J. Park, S. M. Choi, and K. W. Kim. 2001. “The Patterns of Plasma Free Amino Acids after Force-Feeding in Rainbow Trout *Oncorhynchus mykiss* (Walbaum) with and without Dorsal Aorta Cannulation.” *Aquaculture Research* 32 Suppl.:70–75.

Olsen, R. L. and M. R. Hasan. 2012. “A Limited Supply of Fishmeal: Impact on Future Increases in Global Aquaculture Production.” *Trends in Food Science & Technology* 27(2):120–28.

Overturf, K., F. T. Barrows, and R. W. Hardy. 2013. “Effect and Interaction of Rainbow Trout Strain (*Oncorhynchus mykiss*) and Diet Type on Growth and Nutrient Retention.” *Aquaculture Research* 44(4):604–11.

Palmegiano, G. B., F. Daprà, G. Forneris, F. Gai, L. Gasco, K. Guo, P. G. Peiretti, B. Sicuro, and I. Zoccarato. 2006. “Rice Protein Concentrate Meal as a Potential Ingredient in Practical Diets for Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 258(1–4):357–67.

Panserat, S., C. Kolditz, N. Richard, E. Plagnes-Juan, F. Piumi, D. Esquerré, F. Médale, G. Corraze, and S. Kaushik. 2008. “Hepatic Gene Expression Profiles in Juvenile Rainbow Trout (*Oncorhynchus mykiss*) Fed Fishmeal or Fish Oil-Free Diets.” *British Journal of Nutrition* 100(5):953–67.

Refstie, S., O. J. Korsoen, T. Storebakken, G. Baeverfjord, I. Lein, and a. J. Roem. 2000. “Differing Nutritional Responses to Dietary Soybean Meal in Rainbow Trout

(*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*).” *Aquaculture* 190(1–2):49–63.

Ritala, A., S.T. Häkkinen, M. Toivari, and M. G. Wiebe. 2017. “Single Cell Protein—State-of-the-Art, Industrial Landscape and Patents 2001–2016.” *Frontiers in Microbiology* 8:2009.

Rolland, M., B.K. Larsen, J. Holm, J. Dalsgaard, and P. V. Skov. 2015. “Effect of Plant Proteins and Crystalline Amino Acid Supplementation on Postprandial Plasma Amino Acid Profiles and Metabolic Response in Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture International* 23(4):1071–87.

Rumsey, G. L., A. K. Siwicki, D. P. Anderson, and P. R. Bowser. 1994. “Effect of Soybean Protein on Serological Response, Non-Specific Defense Mechanisms, Growth, and Protein Utilization in Rainbow Trout.” *Veterinary Immunology and Immunopathology* 41(3–4):323–39.

Sales, J. 2009. “Prediction of Digestible Energy Content across Feed Ingredients and Fish Species by Linear Regression.” *Fish Physiology and Biochemistry* 35(4):551–65.

Schuhmacher, A., C. Wax, and J. M. Gropp. 1997. “Plasma Amino Acids in Rainbow Trout (*Oncorhynchus mykiss*) Fed Intact Protein or a Crystalline Amino Acid Diet.” *Aquaculture* 151(1–4):15–28.

Stone, D. A. J. 2003. “Dietary Carbohydrate Utilization by Fish.” *Reviews in Fisheries Science* 11(4):337–69.

Sugiura, S. H., J. Gabaudan, F. M. Dong, and R. W. Hardy. 2001. “Dietary Microbial Phytase Supplementation and the Utilization of Phosphorus, Trace Minerals and Protein by Rainbow Trout [*Oncorhynchus mykiss* (Walbaum)] Fed Soybean Meal-Based Diets.” *Aquaculture Research* 32(7):583–92.

Sugiura, S. H., F. M. Dong, and R. W. Hardy. 1998. “Effects of Dietary Supplements of the Availability of Minerals in Fish Meal; Preliminary Observations.” *Aquaculture* 160(3–4):283–303.

Tacon, A. G. J. and M. Metian. 2008. “Global Overview on the Use of Fish Meal and Fish Oil in Industrially Compounded Aquafeeds: Trends and Future Prospects.” *Aquaculture* 285(1–4):146–58.

UN-DESA, 2015. 2015. “World Population Prospects: The 2015 Revision Key Findings and Advance Tables.”

Verdegem, M. C. J., R. H. Bosma, and J. A. J. Verreth. 2006. “Reducing Water Use for Animal Production through Aquaculture.” *International Journal of Water Resources Development* 22(1):101–13.

Weichhart, T. 2012. “Mammalian Target of Rapamycin: A Signaling Kinase for Every Aspect of Cellular Life.” Pp. 1–14 in *Methods in molecular biology (Clifton, N.J.)*. Vol. 821.

Xie, S. and A. Jokumsen. 1997. “Replacement of Fish Meal by Potato Protein Concentrate in Diets for Rainbow Trout, *Oncorhynchus mykiss* (Walbaum): Growth, Feed Utilization and Body Composition.” *Aquaculture Nutrition* 3(1):65–69.

Yamada, S., Simpson, K., Tanaka, Y. and Katayama, T. 1981. Plasma ammo acid changes in rainbow trout force-fed casein and corresponding amino acid mixture. *Bull. Jpn. Sot. Sci. Fish.*, 47: 1035-1040.

Yamamoto, T., Tatsuya U., and T. Akiyama. 1988. “Postprandial Changes in Plasma Free Amino Acid Concentrations of Rainbow Trout Fed Diets Containing Different Protein Sources.” *Fish Physiology and Biochemistry* 64(3):474–81.

Yamamoto, T., Tatsuya U., and T. Akiyama. 2000. “The Influence of Dietary Protein and Fat Levels on Tissue Free Amino Acid Levels of Fingerling Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 182(3–4):353–72.

Yun, H., G. Park, I. Ok, K. Katya, S. Hung, and S. C. Bai. 2016. “Determination of the Dietary Lysine Requirement by Measuring Plasma Free Lysine Concentrations in Rainbow Trout *Oncorhynchus mykiss* after Dorsal Aorta Cannulation.” *Fisheries and Aquatic Sciences* 19(1):4.

Zargar, S., T. S. Moreira, H. Samimi-Seisan, S. Jeganathan, D. Kakade, N. Islam, J. Campbell, and O. A. J. Adegoke. 2011. “Skeletal Muscle Protein Synthesis and the Abundance of the MRNA Translation Initiation Repressor PDCD4 Are Inversely Regulated by Fasting and Refeeding in Rats.” *American Journal of Physiology-Endocrinology and Metabolism* 300(6):E986–92.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Rainbow trout, *Oncorhynchus mykiss*, is the most studied carnivorous fish species in the world as far as nutritional requirements and feed development are concerned. A Google scholar search using the key words ‘rainbow trout nutrition and feeds’ yields over 40,000 citations. The dietary requirements of rainbow trout for essential nutrients are well documented. The need to develop economical sustainable, environmentally-friendly feeds for rainbow trout aquaculture has driven a large increase in research efforts to reduce dependence on feed ingredients derived from marine sources and these efforts have led to a substantial reduction in the use of fishmeal in rainbow trout feeds. However, further reductions will be needed, and evidence suggests that further development of such feeds will require a deeper knowledge of fish physiology, especially digestion and metabolism, and a deeper knowledge of feed ingredients that can be used to provide dietary protein in future feeds. Development of molecular tools will enable fish nutritionists to unravel the complexities associated with nutritional requirements, feed utilization and fish growth efficiency by identifying interrelationships between nutrients, gene expression and cellular metabolism. Fish require the same 40 or so essential nutrients as terrestrial animals, but their aquatic existence and evolutionary history as far as food sources are concerned makes it difficult to utilize the accumulated knowledge of nutrition in poultry or swine, for example, to advance fish nutritional science. Although fish culture has been practiced for millennia, only in the past 60 years has fish nutrition been based on solid nutritional science. Thus, there are many gaps in knowledge in fish nutrition that need to be investigated. What follows is a review of fish nutrition that is focused on the topics that are relevant to this dissertation.

2.2 Amino Acids Requirements

Proteins are the major organic material in fish tissue and carnivorous fish require almost half of their diet to be protein. Proteins are comprised of amino acids

and animals do not have a protein requirement per se, but rather dietary requirements for essential amino acids (Baker, 2008). Thus, the protein requirement of animals corresponds to the requirements for specific essential amino acids (lysine, methionine, threonine, arginine, histidine, isoleucine, leucine, valine, phenylalanine and tryptophan) and to a need for amino groups for the de-novo synthesis of non-essential (dispensable) amino acids such as alanine, asparagine, aspartate, glutamine, glutamate, cysteine, glycine, proline, serine and tyrosine (NRC, 2011). Both essential and non-essential amino acids can be metabolized to produce energy via the tricarboxylic acid cycle (TCA cycle) or, if energy needs are met, to be converted into tissue lipids for future use (Dabrowski and Guderley, 2003). Tissue proteins are comprised of both essential and non-essential amino acids and both participate in other physiological functions in animals. (NRC, 2011). The most common method to determine adequate dietary protein levels has been measurement of weight gain in response to graded increments of dietary proteins (Cowey, 1995; NRC, 2011). The first definitive studies on the protein and amino acid nutrition of fish were made in the late 1950s after the successful development of a purified diet for salmon that was used to determine the qualitative vitamin requirements of fingerling chinook salmon (Halver, 1957). All protein in Halver's diet (about 50% of the diet) was supplied as free amino acids. The free tryptophan content of the diet was 0.7% (or 7g/kg diet). To determine if the amino acid test diet could support normal fish growth, feeding trials were conducted to compare growth of salmon fed the amino acid test diet to growth when dietary protein was supplied by intact protein sources, such as casein and gelatin, the main protein sources used in semi-purified diets used to determine dietary vitamin requirements of salmon. Using a mixture of individual free amino acids present in the same amounts and proportions of the casein-gelatin diet, the amino acid test diet and a casein-gelatin diet were fed to juvenile salmon for 14 weeks. A third experimental diet containing an amino acid mixture that matched that of salmon yolk-sac fry was also fed. The free tryptophan content of this diet was 1.05% (10.5g/kg diet). Salmon grew normally and weight gains were the same between the casein-gelatin diet and the amino acid test diet having an amino acid profile matching that of the casein-gelatin diet. Fish fed the yolk-sac salmon fry amino acid profile diet grew more slowly.

The results of this study demonstrated that growth of salmon was not affected by supplying dietary protein as a mixture of protein concentrate ingredients or a mixture of free amino acids matching the amino acid profile of the casein-gelatin diet. The results also show that the levels of essential amino acids and their proportion in feeds are important determinants of fish growth, even in a diet containing all essential amino acids. Using the amino acid test diet and deleting a single amino acid at a time in feeding studies, the essential amino acids of salmon were identified (Halver and Shanks, 1960). If deletion of a single amino acid had no effect on fish growth, the amino acid was classified as non-essential. If salmon did not grow when a single amino acid was deleted from the amino acid mixture, the amino acid was classified as essential. Salmon were found to require the same 10 essential amino acids as humans and other tested animals. Shanks et al. (1962), following the same methods used in earlier studies with salmon, confirmed that rainbow trout also required the same 10 essential amino acids in the diet.

The quantitative dietary requirements of salmon for essential amino acids were determined using the amino acid test diet to which a single amino acid was supplemented at incremental levels. Typically, weight gain was reduced when levels of the amino acid being tested were below the dietary requirement and leveled off once the dietary requirement level was met. Using this approach, the quantitative dietary requirements of the 10 essential amino acids were estimated. The pioneering studies by Halver and associates, conducted in the 1960s, were published in scientific journals and are summarized in Table 1 of the 1973 NRC Bulletin, “Nutrient Requirements of Trout, Salmon, and Catfish.” Notably, dietary requirements for essential amino acids were expressed as percent of diet (see Table 2.1).

Quantitative dietary requirements for salmon, expressed as a percent of diet, were substantially higher than values reported for chickens, swine and rats, as reported by DeLong et al. (1958). The reasons for this were unclear at the time but are now known to be associated with the natural foods consumed by chickens, swine and rats compared to those consumed by fish, specifically dietary energy sources. Starch is the storage form of energy in plants whereas starch is absent in the foods

fish consume in the aquatic environment. Algae, the primary producers of food in the aquatic environment, store energy as lipids (fats) rather than as starch. As a result, carnivorous species of fish (including salmonids) evolved to derive dietary energy from lipids and protein catabolism. Their ability to utilize carbohydrates as dietary energy sources is limited and feeding high levels of carbohydrates to carnivorous fish such as salmon or trout leads to liver pathologies and reduced growth (Stone, 2003). This is in contrast to the capacity of terrestrial animals and birds to utilize dietary starch as an energy source.

Experiments regarding protein or amino acid requirements usually are conducted on young, rapidly growing fish because the maintenance requirements at this life stage constitute a small proportion of the total requirements for maintenance and growth (Cowey, 1995). The first diets used to determine gross protein requirements contained a mixture of casein, gelatin and crystalline amino acids combined to simulate the amino acid content of whole chicken-egg protein. This diet type is considered purified test diet which means that all its components are refined and have a precisely defined composition (Wilson and Halver, 1986). Apart from the purified diet, two other types of test diets are used, i.e., the semi-purified and the practical. Semi-purified diets contain some natural ingredients like corn gluten in a relatively pure form in combination with purified ingredients, while practical diets are formulated using mainly practical ingredients such as fishmeal, protein concentrates and animal by-products. In any case, when these diets are used as test diets for amino acid requirements, they must be formulated with a fixed amount of protein which should be below the required level with remaining protein needs supplied in the form of crystalline amino acids (Wilson and Halver, 1986). However, as mentioned, fish do not have a specific protein requirement *per se* but rather defined requirements for essential and non-essential amino acids that constitute proteins. Many studies for a large number of fish species have been conducted with the objective of estimating the minimum amino acid requirements for optimal growth (NRC, 2011). Two assumptions are made when amino acid requirement studies are performed. First, there is a dietary level of each amino acid that, when reached, satisfies the physiological needs of the

animal under study. Second, amino acids in the experimental diets are considered fully utilized, i.e. 100% digestible.

Even though live weight gain is the most common response variable in amino acid requirement studies, other response variables or assessment methods have also been used including direct and indirect oxidation studies, tissue pathology, concentration of free amino acids in systemic blood and muscle tissue, the ideal protein concept and the A/E ratio concept (Lall and Andreson, 2005; NRC, 2011).

Oxidation studies are categorized as either direct and indirect methods and both are applied at the end of dose response feeding trials. In the direct oxidation method, a $^{14}CO_2$ production measurement, involves the response to an intraperitoneally injected pulse of a ^{14}C-labelled amino acid being tested. The principle of this method is based upon the minimum oxidation degree when the dietary level of the amino acid tested meets the requirements (Cowey, 1995). In the indirect method a dispensable amino acid is ^{14}C-labelled, added to the diet the fish is consuming and $^{14}CO_2$ production is measured. The principle of this method is based upon the law of the minimum which implies that when the requirements of the amino acid under study are not met then other dispensable amino acids in excess are catabolized (NRC, 2011). Amino acid requirement estimation based upon tissue pathology has also been proposed for certain amino acids that cause distinct pathological signs when deficient in the diet. When a dietary level for such an amino acid is met, the pathology can no longer be detected, and this level is estimated as the dietary requirement. A good example is tryptophan deficiency, which is reported to cause lordosis, scoliosis, cataracts and caudal fin erosion (Walton et al., 1984). Methionine deficiency in rainbow trout is reported to cause cataract formation (Cowey, 1995). Blood and muscle free amino acid concentrations are response variables that have gained an increased interest as a more robust method of estimation of the requirement for essential amino acids because this approach relates dietary intake to protein deposition (Kaushik and Luquet, 1979). This method is based upon the fact that tissue concentrations begin to increase once the amino acid dietary level exceeds the requirement. It refers to a situation where all essential amino acids are co-limiting for

performance so that the amino acid supply exactly matches the amino acid requirements, the so-called ideal protein (Furuya et al., 2004). The requirements for amino acids in an ideal protein are usually expressed relative to the requirement for lysine (Milgen and Dourmad, 2015). Lysine is used as the reference amino acid because it is almost exclusively utilized for body protein accretion and thus not confounded by its use in various metabolic pathways for maintenance or production (Furuya et al., 2004).

The A/E ratio is a method of estimation that resembles the ideal protein concept because it simulates the whole-body amino acid composition but differs by attempting to estimate simultaneously all the 10 essential amino acids. The A/E ratio is defined as [(each essential amino acid content / total essential amino acid content including cystine and tyrosine) x 1000] (Small and Soares, 1998).

However, most of the protein requirement values in finfish species have been obtained using a dose response curve of weight gain and they show large variations in the estimated requirements (Lall and Anderson, 2005). Regarding quantitative amino acid requirements, many studies for a large number of species have been conducted with the objective estimating the minimum amino acid requirements for optimal growth. Results showed marked discrepancies many times for the same amino acid in a single species (Bureau and Encarnacao, 2006). Variations in the essential amino acid requirements of fish can be attributed to a number of factors such as differences in basal diet composition, size and age of fish, growth rate achieved, genetic differences, method of feeding (fixed rate vs satiation feeding) and culture conditions, response criteria, as well as differences in the mathematical and statistical approaches used to analyze data, all of which affect overall growth rate besides experimental design and choice of response criterion (Lovell, 1988; Bureau and Encarnacao, 2006).

2.3 Digestion

Digestion refers to the process of degradation of structurally complex food components into smaller molecules which can be transported across the intestinal

epithelium by the action of digestive enzymes and other components produced by the digestive system to support physiological processes. The generalized digestive system of fish includes the mouth, esophagus, stomach, pylorus, pyloric caeca, intestine and associated organs like liver, gall bladder and pancreas. The components secreted from the stomach, liver (via the gall bladder) and exocrine pancreas are of major importance for enzymatic hydrolysis of complex food polymers, namely proteins, carbohydrates and fats into smaller fragments (Bakke et al., 2010). Digestion begins from the mouth and the pharynx with the mechanical breakdown of food without digestion of digestive enzymes (Rust, 2003). Once food enters the stomach its presence stimulates increased secretion of hydrochloric acid and pepsinogen by the oxynticopeptic cells. The acidic conditions promote the conversion of pepsinogen to pepsin. The chyme, which is the flow digesta, is partially digested food which contains dissolved nutrients such as proteins and polypeptides, fats, oligo and polysaccharides, vitamins and minerals, enters the intestine stimulating secretions from the gall bladder and pancreas and the intestine itself. These secretions include bicarbonate, which is neutralizing the acidic pH of the chyme, bile acids (liver origin via gall bladder) which are aiding the digestion of fats and various enzymes of pancreatic origin such as proteases (trypsin, chymotrypsin, elastase, carboxypeptidase, aminopeptidase and peptidase), lipases (pancreatic lipase and colipase) and glucosidases (amylase) (Rust, 2003).

2.3.1 Protein Digestion

The gastric process yields partially denatured and hydrolyzed proteins and peptides in an acid solution passing to the upper intestinal compartment for further hydrolysis by proteases which are enzymes with proteolytic activity. The end products of protein hydrolysis are tripeptides, dipeptides and free amino acids, molecules which have molecular sizes suitable for transport across the brush border membrane of intestinal epithelial cells. The different proteases are characterized according to their mode of cleaving action. Trypsin, chymotrypsin and elastase are endopeptidases meaning that the peptide bond for cleavage is positioned inside the polypeptide chain.

In the category of exopeptidases (peptide bond either at the C- or N-terminus) are the carboxypeptidases and aminopeptidases (Pizauro et al., 2004).

Hydrochloric acid secretion in the stomach is responsible for protein denaturation, the first step in protein digestion. Pepsinogen is a zymogen that is activated by acidic pH and it is converted to pepsin, which in turn starts the active protein hydrolysis process as an endopeptidase by cleaving internally the protein peptide bonds formed by phenylalanine, tyrosine or leucine (Zhao et al., 2011). After this initial protein denaturation and digestion, further digestive activity takes place in the intestine. All enzymes of the intestine are also zymogens and their processing begin with the activation of trypsin by the action of an enteropeptidase called enterokinase (Pizauro et al., 2004). Trypsin, chymotrypsin and elastase are endopeptidases and also belong to the group of serine proteases. Once trypsin is active, all the other zymogens are also activated by its action (Pizauro et al., 2004). Trypsin cleaves the carbonyl group of arginine and lysine. Chymotrypsin cleaves next to tryptophan, tyrosine, phenylalanine, leucine and methionine. Elastase cleaves next to alanine, glycine and serine. Carboxypeptidases A and B are exopeptidases and they are considered metalloenzymes because they depend on zinc for their catalytic activity. Carboxypeptidase A has a cleavage specificity for alanine, isoleucine, leucine and valine and carboxypeptidase B has cleavage specificity for arginine and lysine. Located at the intestinal surface are the aminopeptidases and dipeptidases. The aminopeptidases are considered non-specific exopeptidases which cleave repeatedly at the N-terminal of amino acids, while the dipeptidases liberate free amino acids by cleaving dipeptides (Rust, 2003).

After protein digestion the final products consists of tri- and di-peptides and free amino acids which are in a form that can be transported by the intestinal epithelial cells into the blood stream. The main transport occurs transcellularly through a sodium-dependent system, secondary system and co-transport, in the case that the luminal concentrations are very high paracellular diffusion may occur (Sundell and Ronnestad, 2011). The proximal area of the intestine is considered the major site of peptide and amino acid absorption but other parts of the small intestine also have

transport activity (Dabrowski, 1986). Oligopeptides and free amino acids are absorbed along the intestinal tract by specialized membrane transporter proteins. In animals, free amino acids are transported by a variety of sodium-dependent and-independent membrane transporters (Broer, 2008). Amino acid transporters have been classified into distinct systems dependent upon substrate specificity, transport mechanism and regulatory properties (Hyde et al., 2003). The 20 major amino acids can be divided into neutral, basic, acidic and imino acids and in an initial work by Halvor Christensen (1989) in non-epithelial cells showed that amino acid transport systems accept groups of amino acids rather than individual ones (Broer, 2008). Christensen's work defines 'system L' as the system that was responsible for the transport of leucine and other hydrophobic neutral amino acids; 'system A' for alanine and other small polar and neutral amino acids; and 'system ASC' for alanine, serine and cysteine. A separate nomenclature has also been applied for systems mediating transport of anionic amino acids (X_{AG}) and cationic amino acids (y_), while the lowercase acronyms indicate Na_-independent transporters, uppercase acronyms are used for Na_-dependent transporters (Brower 2008). Tri- and di-peptides are transported via a specialized proton oligopeptide co-transporter, PEPtide Transporter 1 (PEPT1), which is located in the brush-border membrane of epithelial intestinal cells (Verri et al., 2011). In fish, essential amino acids generally show higher uptake rates than non-essential amino acids and the affinities of the sodium dependent transporters are generally higher than the corresponding mammalian ones. This is thought to be due to the relatively short intestinal length in fish compared to mammals (Sundell and Ronnestad, 2011).

Amino acids which are absorbed by the brush-border membrane of the intestinal epithelial cells are transported to the blood via transporters located at the basolateral membrane of the cells. The hepatic portal vein carries them to the liver where they can have different fates depending upon the physiological status of the organism and their relative quantity (quantity of essential and non-essential amino acids) (Jurss and Bastrop, 1995). Protein turnover refers to the continual renewal or replacement of protein. It is defined by the balance between protein synthesis and protein degradation (Hinkson and Elias, 2011). When an animal is in protein balance, defined as the state where protein synthesis is equal to protein degradation, then

protein turnover is equal to protein synthesis or degradation (Fraser and Rogers, 2007). In fish, high protein retention indicates reduced protein degradation rates and hence low protein turnover rates (Houlihan et al., 1995).

It is very important to take into consideration tissue-specific activity in terms of the protein turnover. Although, in absolute terms, protein in intestine and liver tissues represent a small portion of the whole-body protein content, their turnover activity is much higher than that of the muscle tissue which constitutes the highest percentage of whole-body protein (Fauconneau and Arnal,1985). Fauconneau and Arnal (1985), using rainbow trout, showed that muscle accounts for only approximately 33% of whole-body protein synthesis when fish were held at 10°C. By monitoring the turnover selectively at different body sites, a better view of the dynamic response of the organism to the diet can be obtained (Skinner et al., 2017. In short, the differences observed in those organs should be much larger than those observed in muscle. The small intestine is the primary organ responsible for terminal digestion and absorption of dietary protein and amino acids. Even though for many years it was thought that intestinal amino acid catabolism was related to the non-essential amino acids as substrates for provision of energy, recent research findings proved that this is not entirely true (Wu et al., 2013). In fact, essential amino acids are being used for mucosal protein synthesis and are also catabolized prior to post intestinal processing, more specifically, for the case of lysine, methionine and threonine, the three most commonly added amino acids (Wu, 1998). Results of recent studies indicate that enterocytes can degrade BCAA, but oxidation of lysine, methionine, phenylalanine, threonine and histidine to CO_2 is absent or negligible from enterocytes (Wu et al., 2013). However, intestinal bacteria can degrade all amino acids and are primarily responsible for the catabolism of lysine, methionine, phenylalanine, tryptophan, threonine and histidine in the small intestine (Dai et al., 2012). Therefore, amino acids should be considered essential also for the microbiota harbored in the gut of every organism and may have a role in shaping the latter, thereby affecting the host eventually (Zhao et al., 2018). When proteins are hydrolyzed, free amino acids are released and absorbed in the various tissues. A portion of these amino acids may be promptly utilized for the synthesis of proteins, whereas a proportion maybe

deaminated and used for energy production. Amino acids had been classified traditionally as nutritionally essential or non-essential based on growth or nitrogen balance of animals (Wu, 2009). Dietary amino acids are required by animals primarily for maintenance and protein accretion. Components of amino acid maintenance include: a) protein synthesis; b) their obligatory use as precursors of essential metabolites; c) their obligatory oxidation; d) their use by gastrointestinal epithelia and luminal microbes; and e) their use from the integumentary system (Wu et al., 2013). If a diet balanced in essential amino acids is provided, amino acid oxidation should be minimized. The major consequence of inadequate protein intake or diets lacking in specific essential amino acids relative to others (amino acid imbalance) is a shift in this balance, so that rates of synthesis of some body proteins decrease while protein degradation continues, thus providing an endogenous source of those amino acid most needed. There are two major multi-enzyme systems of protein degradation, the lysosomal and the proteasomal (Seiliez et al., 2014). The ubiquitin-proteasome system is highly selective, so it can account for the wide range of degradation rates (half-lives raging from minutes to hours up to days) observed for different proteins (Zhang et al., 2007). It is thought to be primarily responsible for degrading abnormal or damaged proteins, along with regulatory proteins that are typically synthesized and degraded very rapidly (Peters, 1994). Lysosomes are membrane-enclosed vesicles inside cells that contain a variety of proteolytic enzymes and operate mostly at acidic pH (Appelqvist et al., 2013). This system is thought to be unselective in most cases, although it can also degrade specific intracellular proteins (Nakamura and Yoshimori, 2017). This system is found to be highly regulated by hormones such as insulin and glucocorticoids, and also by amino acids (Chen et al., 2017; Seiliez et al., 2012).

2.4. Alternative Proteins

Plant protein products are considered the main alternatives to fishmeal and their inclusion in aquafeeds has increased because they are considered sustainable and economical viable sources of protein (Hardy, 2010). This is in contrast to the situation with fishmeal production, which cannot increase beyond current levels, and where global demand exceeds global supply, resulting in rising and unstable prices

(Hardy, 2010). The main categories for ingredients that are considered alternative to fishmeal protein sources are the cereal grains (corn, wheat, rice, barley and sorghum), the oilseeds (soybean, canola meal, sunflower and cottonseed) and the pulses (lupins, filed beans and fava beans) (Gatlin et al., 2007). Cereal grains and oilseeds are grown primarily to produce products for human consumption; animal, poultry and fish feed ingredients are produced from the residue remaining after primary products are removed (Awika, 2011).

All plant protein products present challenges as fish feed ingredients for three main reasons: 1) their essential nutrient content, most importantly essential amino acid content, do not match the dietary requirements of fish; 2) they contain compounds that reduce feed intake, interfering with digestion or cause physiological problems; and 3) they do not contain many of the biologically-active compounds found in fishmeal (Francis et al., 2001; Gatlin et al., 2007; Hardy, 2010). The cereal grains are considered the most important crops in the world (Awika, 2011). However, cereal grains contain substantially less protein than oilseeds or pulses, making it necessary to further process grain and grain byproducts to produce protein concentrates which can then be used as protein sources in fish feeds (Freer and Dove, 2002). Similarly, pulses must be further processed to produce suitable protein concentrates for fish feeds (Overland et al., 2009). Oilseeds, in contrast, are grown to produce oils, their primary product, for human consumption (Carrier et al., 2012). The residue remaining after oil is removed from oilseeds is the base material from which protein concentrates are produced. Compared to legume seeds, oilseeds containing less protein (Moss and Baudet, 1983).

2.4.1 Cereal Grains

Cereal grains are considered the most important crops in the world. Corn, wheat and rice account for over 50% of world's daily caloric intake forming the basis for most foods six billion humans on earth consume (Awika, 2011). However, because cereal grains contain substantially less protein than oilseeds or pulses, ingredients of cereal grain origin are used as concentrates by the aquafeed industry (Shewry and Halford 2002).

2.4.1.1 Corn Gluten Meal

One of the primary products of corn is corn oil for human consumption (Ai and Jane, 2016). Corn oil is produced using the wet milling process that partitions the kernel into starch, oil, corn gluten meal and corn gluten feed. Corn gluten meal is a protein concentrate product with over 60% protein content guaranteed and the primary corn product used as a protein source in fish feeds (Yigit et al., 2012). Corn gluten meal protein is highly digestible but contains low levels of lysine (Gatlin et al., 2007). Another limiting factor for the use of corn gluten meal is its xanthophyll content. Farmed fish having white flesh, especially rainbow trout, exhibit yellow fillet pigmentation when feeds contain xanthophyll found in corn gluten meal. The yellow colored flesh lowers its market value to consumers (Francis et al., 2001).

2.4.1.2 Wheat Gluten Meal

Flour is the primary product produced from wheat, and flour is primarily starch, the storage form of energy in most grains. Vital wheat gluten is the separation product from wheat starch by the wet milling process and has a minimum guaranteed protein content of 75% (Apper-Bossard et al., 2013). Wheat gluten is considered one of the best plant protein concentrates to use in fish feed due to its high protein digestibility and the fact that it lacks antinutritional factors, with the exception of phytic acid (Hardy, 1996). There are only two major problems with its use. First, its amino acid profile is considered inferior to fishmeal in lysine, tryptophan, and arginine (Apper-Bossard et al., 2013). Second and most importantly, even though it is a sustainable source of protein it is also the most expensive among the plant proteins (Hardy, 1996).

2.4.1.3 Rice

Rice protein concentrate is a by-product of rice syrup manufacturing. It is the remaining fraction of rice after it undergoes an enzymatic process so the complex carbohydrates are transformed into sugars which are removed as rice syrup. Its protein content is 70% but it is low in lysine as are all the cereal grain proteins (Palmegiano et al., 2006).

2.4.2 Pulses

Pulses are grain legumes that are widely used animal nutrition and recently have been used in aquafeeds (Allan, 2000). Pulses have been cultivated for millennia and have become essential foods for humans and for use in animal feeds, while are also playing an important role in cropping patterns of farming because they add nitrogen to the soil (Calles, 2016). Globally, pulse production increased from 44.9 million tonnes in 1981-1983 to 72.3 million tonnes in 2011-2013 (Sherasia et al., 2017). Pulse proteins originate from edible seeds of legumes (plants with a pod), which include dry peas, beans, lentils and chickpeas. Pulses contain 17–30% of protein, and the major proteins found in pulses are globulins (legumin and vicilin) and albumins (Jahan-Mihan et al., 2011).

2.4.2.1 Lupins

Lupin seed meal is a product of wet extraction. Lupin seed meal has a protein content of 30-40% (Pisarikova and Zraly, 2009). From a nutrient point of view, lupin meal has considerable potential to replace fishmeal in fish feeds. However, the low lysine and methionine content of lupin proteins and the presence of antinutritional factors such as alkaloids (affecting feed intake), and oligosaccharides which could affect nutrient digestibility, present challenges to high inclusion levels of lupin meals in fish feeds (Gatlin et al., 2007).

2.4.2.2 Peas

Pea protein concentrate is produced by air classification subsequent to dry fractionation by fine grinding of dehulled peas. Pea protein concentrate contains 50% protein making it suitable for use in aquafeeds (Thiessen et al., 2003). Similar to lupin meal, pea protein concentrate is characterized by a low level of lysine and methionine and it contains the same antinutritional factors (Gatlin et al., 2007).

2.4.3 Oilseeds

Oilseed meals are produced from the residue, or cake, remaining after oils for human food use are removed. They play an enormously important role in feeds for

livestock, poultry and fish (McKevith, 2005). The main products of this category are soybean meal, soy protein concentrate, canola / rapeseed meal, sunflower meal and cottonseed meal. Soy proteins are derived from soybeans, which have high protein content (35-40%), the major part of the proteins in soybeans existing as storage proteins, primarily β-conglycinin and glycinin (Yaklich, 2001). Regarding the proteins from the other oilseed plants, the biggest fraction of proteins is represented by storage proteins, including cruciferin in canola or rapeseed, zein in corn, 11S protein in cottonseed, 12S protein in flax, carmin in safflower and helianthin in sunflower (Arntfield et al., 2004).

2.4.3.1 Soybean Meal

The most common soybean meal product in the aquafeeds is solvent-extracted soybean meal (Hardy, 2010). It is a product made from dehulled soybeans that are subjected to solvent extraction to remove soy oil, the primary product for human use. The soy cake is then toasted and ground to produce soybean meal. Defatted, dehulled soybean meal contains a minimum of 48% protein (Stein et al., 2008). In regard to its amino acid profile, soybean meal is deficient in lysine, methionine and threonine. However, the biggest limitation in the use of soybean meal in aquafeeds is related to its content of antinutritional factors such as protein inhibitors (trypsin inhibitor), non-starch polysaccharides, oligosaccharides (raffinose and stachyose), lectins (agglutinins), antigenic proteins (glycinin and β-conglycinin), phytoestrogenic compounds (daidzein and genistein), phytic acid and saponins (Francis et al., 2001).

2.4.3.2 Soy Protein Concentrate

Soy protein concentrate is produced by aqueous alcohol extraction of the defatted white soy flakes that removes most of the non-soluble polysaccharides and other carbohydrate fractions. Its minimum guaranteed protein content is 60% but its high protein content is offset by relatively low levels of methionine and lysine (Day and Gonzalez, 2000). However, regarding its antinutritional factor content, the aqueous alcoholic extraction process removes the soluble carbohydrates and significantly

reduces the levels of all the other antinutritional factors which are present in soybean meal except for phytic acid (Francis et al., 2001).

2.4.3.3 Rapeseed Meal/Canola Meal

Rapeseed/canola meal is produced by solvent extraction of oil, again the primary product, followed by drying and grinding the cake. The meal contains 35-40% protein (Little et al., 2015). Canola is a patented line of cultivars of rapeseed that have been bred to contain low levels of erucic acid and glucosinolates, both of which are antinutritional factors (Enami, 2011). Its amino acid profile is similar to that of soybean and it is deficient in lysine (Jiang et al., 2018).

2.4.3.4 Sunflower Meal

Sunflower meal is produced from the dehulled sunflower seeds following oil extraction. As with other oilseeds, the oil is the primary product and the meal is a byproduct. Its protein content is around 44% but is low in lysine content (Lovell, 1998). Sunflower meal is low in antinutritional factors (Martinez et al., 2015). The main impediment to its high inclusion in aquafeeds is related to its relatively high fiber content (Lovell, 1998).

2.4.3.5 Cottonseed Meal

Cottonseed meal is produced by solvent extraction with a general protein content of 41% (Lovell, 1998). Is considered deficient in lysine and but also its methionine content is considered somewhat low (Li and Robinson, 2006). Its major antinutritional factor is gossypol which is a cause of depressed growth and anorexia (Gatlin et al., 2007).

2.5 Fishmeal Replacement Studies

The scientific literature contains thousands of papers describing the effects of replacing a portion of fishmeal in fish feeds with alternative proteins on fish growth performance, feed efficiency, fish health and numerous other response variables.

What follows are summaries of published studies that most directly relate to the research undertaken in this dissertation.

Sanz et al. (1994) conducted a nutritional experiment using rainbow trout (40 g). In the experiment, three diets were fed to rainbow trout for 45 days consisting of a control diet (fishmeal-based) and two other diets formulated with 40% of the dietary protein provided from either soybean meal or sunflower meal. The results from the experiment showed no significant differences on weight gain. The soybean meal diet and the sunflower diet showed higher protein digestibility coefficients compared to fishmeal diet. The soybean meal diet showed higher palatability based upon feed intake. In a trial conducted by Kaushik et al. (1995), rainbow trout (83 g) were fed diets containing graded levels of either soy flour or soy protein concentrate supplemented with methionine as partial or total replacement of fishmeal for 12 weeks. Their results showed soy protein concentrate could replace 100% of the fishmeal with no effect on growth performance or nutrient utilization. In contrast, more than 50% fishmeal replacement with soy flour reduced growth rate and feed efficiency. Plasma cholesterol levels were significantly reduced in fish fed diets containing soy flour or soy protein concentrate. Refstie et al. (2000) compared the responses of rainbow trout and Atlantic salmon being fed diets containing defatted soybean meal. Salmon and trout (200 g and 100 g, respectively) were fed either a fishmeal-based diet (LT quality) or a diet containing 32% fishmeal and 30% soybean meal for a 12-week period. The results showed trout performed equally well when fed both diets whereas salmon gained 44% more weight on the fishmeal diet compared to the diet containing soybean meal. For both species, digestibility values were higher for fat and energy for the fishmeal diet, while nitrogen digestibility was not affected by diet. Nitrogen and energy retention were higher in fish fed the fishmeal-based diet. However, both species developed enteritis in the distal intestine when fed the diet contained 30% soybean meal. Yamamoto et al. (2000) using rainbow trout fingerlings (1.1 g) tried to evaluate growth and estimate the quantitative adequacy of amino acids in practical dietary proteins by analyzing the free amino acid levels in various tissues of rainbow trout fed a fishmeal based diet, a soybean based diet (67% SBM), a malt protein flour-based diet (62% MPF) or a diet in combination of the soybean meal and malt protein meal

for 9 weeks. The fish groups fed all three plant-based diets showed decreased growth performance. Further, all the plant diet fish groups showed that free amino acids of various tissues were highly correlated with the protein source amino acid profile and in particular the levels of lysine, methionine and threonine were quite lower in fish fed the plant protein diets compared to the fish fed fishmeal-based diet.

Romarheim et al. (2006) compared solvent-extracted, toasted soybean meal and untoasted soy white flakes as partial replacers of fishmeal in rainbow trout diets. Rainbow trout, 300g initial weight, were fed three diets; a control diet (49% fishmeal), a toasted soybean diet (29% FM and 25% SBM) and an untoasted soybean white flake diet (29% FM and 25%SBM) for a period of 63 days. The fish fed the diets with either toasted or untoasted soybean meal, compared to fishmeal diet, showed reduced growth, lower feed intake, higher feed conversion ratio, lower nitrogen retention, lower plasma cholesterol and triacylglycerols, lower ADC values for fat and amino acids, lower leucine amino peptidase activity and higher trypsin activity.

Teskeredzic et al. (1995) evaluated three sources of rapeseed meal as partial or total replacements of fishmeal in practical diets of rainbow trout. Rainbow trout juveniles (4.3 g) were fed ten diets consisting of a control diet (fishmeal-based), and experimental diets containing three rapeseed meal sources (undephytinized as untreated control, solvent-treated undephytinized and dephytinized) at three fishmeal-protein replacement levels (33%, 66% and 100%). The results showed that fish groups fed either the untreated control or the dephytinized rapeseed meal at 66% fishmeal-protein replacement in the diet did not differ from the control diet fed group in growth rate, feed intake, feed efficiency, protein and gross energy utilization, survival and health. However, the fish fed undephytinized untreated at 33% fishmeal protein replacement, showed the highest growth, feed efficiency and protein utilization. Xie et al. (1997) evaluated graded levels of potato protein concentrate in diets of rainbow trout as a replacement for fishmeal. Rainbow trout with an average weight 4.77 g were fed for 6 weeks, five experimental diets consisting of a control fishmeal-based diet and experimental diets containing graded levels potato protein concentrate (17.5%, 37%, 56% and 100%). At the end of the trial, the results showed that incorporation of potato

protein concentrate led to a linear decrease in performance on all the parameters including growth, feed efficiency and protein efficiency. Whole body dry matter, protein and fat also decreased with increasing levels of potato protein concentrate in the diet. Thiessen et al. (2004) conducted two experiments (nine weeks each) using dephytinized canola protein concentrate as a replacement for fishmeal. The results from the first experiment showed that canola protein concentrate could replace up to 75% of fishmeal protein without any negative effect on performance of rainbow trout (28 g initial weight). The second experiment showed that canola protein concentrate could replace up to 30% of the fishmeal protein in diets containing fishmeal, soybean meal and corn gluten meal without negatively affecting growth performance and nutrient utilization of post-juvenile rainbow trout (179 g) rainbow trout. Palmegiano et al. (2006) evaluated rice protein concentrate (RPC) as a fishmeal substitute in the diets of rainbow trout. Rainbow trout (62.4 g) were fed diets containing incremental levels of RPC (0, 20, 35 and 53%) for a period of 94 days. The results showed that inclusion of RPC higher than 20% resulted in an almost linear reduction in the apparent digestibility coefficients (ADCs) of nutrients and energy and this was mirrored by the growth performance of the fish.

Escaffre et al. (2007) studied intestinal and liver histology of rainbow trout when the fish were fed a diet in which fishmeal was replaced completely by soy protein concentrate in high energy diets (23 MJ/Kg GE). Rainbow trout, initial weight 106 g, were acclimatized for 90 days on a fishmeal-based or a SPC-based diet and then for 14 days were fed 1.0 g kg BW^{-1} d^{-1} and six hours from the last meal 10 fish were sampled for histological analysis. The results showed no diet difference regarding fold height, epithelium length, stroma proportion and number of cells infiltered between the enterocytes in the proximal and distal intestines. In contrast enterocyte height and width were lower in SPC-fed fish in the distal intestine and the mean hepatocyte volume was on an average 36% lower in SPC-fed fish and was positively correlated to the hepatosomatic index for fish fed this diet only. The authors concluded that SPC did not cause inflammatory reaction of the gut nor did it affect the epithelium surface. Slawski et al. (2012) conducted a study to evaluate a high-quality rapeseed protein

concentrate (71% crude protein) as a fishmeal replacement on growth performance, feed efficiency, blood parameters and histological morphology in rainbow trout. Rainbow trout, initial weight 37.8 g, were fed three diets in which a high-quality rapeseed protein concentrate replaced fishmeal at two replacement levels (0%, 66% and 100%). At the end of the 84-day experimental period the results showed no difference between the total fishmeal replacement diet fed group and the control for fish weight gain, feed intake, feed conversion ratio and survival. The inclusion of rapeseed protein concentrate decreased the ash content but did not affect other proximate constituents in the whole trout body. Regarding blood parameters and intestinal morphology, the inclusion did not affect any of the parameters investigated.

De Francesco et al. (2004) performed a study to evaluate the long-term effect that plant protein-based diet had on fish growth, morphological and body quality traits of rainbow trout. Post-juvenile rainbow trout, initial weight 162.5 g, were fed either a fishmeal-based diet or a diet containing a blend of plant protein concentrates that replaced fishmeal (corn gluten, wheat gluten, extruded peas and rapeseed meal) for a period of 24 weeks. Compared to the fishmeal group the fish fed with the plant protein-based diet showed a lower growth rate, feed efficiency and protein efficiency ratio even though feed intake did not differ significantly between dietary treatment groups. Moreover, fish fed the plant protein-based diet had a lower dressed weight and fillet lipid content. Volhelmsson et al. (2004) investigated growth and metabolism in rainbow trout (19 g) fed either a plant protein-based diet (corn gluten, wheat gluten, extruded peas and rapeseed meal) or a fishmeal-based diet. To measure effects of diet on metabolism they used a liver proteomic approach (2-D). After a period of 12 weeks the fish group fed the plant-based diet showed a significant decrease in growth rate, feed efficiency and protein efficiency compare to the fishmeal diet. The proteomic study showed that the plant-based diet affected pathways involved in primary energy generation (increased levels of NADPH and malic enzyme), maintenance of reducing potential (increased levels of a subunit of an electron transferring flavoprotein and increased levels of cytochrome c oxidase), bile acid synthesis (increased levels of H-fatty acid binding protein), and cellular protein degradation (increased levels of

proteasomes). Barrows et al., (2007) performed a feeding trial testing diets with three different protein sources (fishmeal and barley protein), plant concentrates (rice protein, soy protein concentrate and barley protein) and plant meals (wheat gluten, corn gluten and soybean meal). Using two different nutrient densities; a high (48% protein and 18% fat) and low (43% protein and 13% fat), and evaluate their effects on growth efficiency, nutrient digestibility and plasma amino acid concentrations on rainbow trout (initial weight 38 g). Their results showed protein source and nutrient density affected feed intake, weight gain and feed conversion ratio with the high-density diets positively affecting and the fishmeal-barley diet performing 10% better than the other two protein sources diets. Regarding the apparent digestibility coefficients and plasma amino acid availabilities, they also corresponded to the differences recorded in weight gain, while protein retention was affected only by the protein source with the fishmeal-based diet showing the highest value. The authors concluded that fishmeal-free diets using conventional and concentrated plant protein ingredients, are good but some limitations to growth exists compared to fishmeal diets.

Panserat et al. (2008) evaluated the effect of dietary fishmeal replacement by a plant protein blend (white lupin, corn gluten, wheat gluten, dehulled peas and soybean meal) on growth performance and on the hepatic transcriptome (cDNA microarrays 9K) in rainbow trout (initial average weight 0.21 g, first feeding). After a period of 52 weeks, the fish group fed the plant-based diet showed lower weight, lower feed efficiency and lower protein feed efficiency than fish fed the fishmeal diet even though feed intake was higher in fish fed the plant-based diet. Regarding the hepatic transcriptome, analysis showed an alteration of expression of 75 genes, mainly genes related to lipid metabolism, amino acid metabolism, protein biosynthesis, protein transport, transcription regulation, generation of energy and signal transduction.

Collins et al., (2012) tried to assess the effects of increasing inclusion rates of plant protein in the diets on growth of rainbow trout. In a series of six experiments, they used soybean meal (SBM), soy protein concentrate (SPC), pea meal (PM), pea protein concentrate (PPC), canola meal (CM) and canola protein concentrate (CPC) at the following inclusion rates (0%, 7.5%, 15%, 22.5% and 30%) while maintaining

the level of fishmeal as constant as possible (~33%). Linear and quadratic regression equations of ingredient inclusion levels on average daily feed intake, specific growth rate, feed conversion ratio and protein efficiency ratio were calculated. The results did not show any significant negative effects on the parameters tested at any dietary level of SPC, CPC, PM and PPC. In contrast SBM and CM showed significant negative effects on specific growth rate, feed conversion ratio and protein efficiency ratio. Burr et al. (2012) conducted a study to evaluate blends of alternate proteins as replacements for fishmeal in diets for rainbow trout. Rainbow trout with initial average weight of 19.5 g were fed ten experimental, practical diets consisting of a control fishmeal-based diet, and three plant protein concentrates (soy protein concentrate, corn gluten meal and barley protein concentrate) at three inclusion levels in order to replace fishmeal by 63%, 82% and 100%. After a 12-week period, the results showed the fish groups fed the soy protein concentrate-based diet at 63% and 82% did not differ in weight gain from the control group but weight gain was reduced in fish fed the diet in which 100% of fishmeal was replaced.

Nagel et al. (2012) examined the potential of two protein concentrated fractions of rapeseed, namely albumin and globulin to replace fishmeal in rainbow trout diets. Fish having an initial weight 31.5 g were fed diets formulated to contain graded levels of either albumin or globulin (0, 50, 75 and 100%) on a digestible protein basis for 10 weeks. The results showed that growth of fish fed the diet with 50% albumin performed equal to fish fed the fishmeal diet while a higher inclusion of albumin as well as any inclusion of globulin (due to higher levels of glucosinolates and sinapinic acid) reduced growth and survival. Regarding whole-body composition, albumin inclusion lowered the fat content of the fish, while globulin at 75 and 100% inclusion significantly reduced the crude protein content of the fish.

A number of research studies have been conducted to assess the effects of reducing phytic acid levels in soybean meal to improve its nutritional value. Sugiura et al. (2001) conducted four series of experiments in order to evaluate the effects of thermal and enzymatic (by phytase) treatments of soybean meal on apparent digestibility coefficients of total-phosphorus, phytate-phosphorus, protein, ash,

calcium, magnesium, copper, iron, manganese, strontium and zinc in rainbow trout. In the first experiment, fish fed six experimental diets characterized as high-ash (soybean meal 30% and fishmeal 42%) consisting of thermally untreated soybean meal, soybean meal heated in a microwave oven, soybean meal dry-roasted, soybean meal heated in a pressure cooker without adding water, soybean meal heated in a pressure cooker with added water and soybean meal treated (1000 U/Kg of diet phytase). In the second experiment, the fish were fed three high-ash diets containing thermally untreated soybean meal with the addition of either phytase (1000 U/Kg diet), citric acid (5% of diet) or both (phytase 1000 U/Kg and 5% citric acid). During the third experiment, fish were fed five low-ash diets (formulated to contain untreated soybean meal at 50% of diet) with incremental concentrations of phytase (0, 500, 1000, 2000 and 4000 U/Kg diet on dry basis) and a sixth low-ash diet but the soybean meal was phytase-treated (200 U/Kg of soybean meal). In a fourth experiment, the fish were fed three low-ash diets containing untreated soybean meal with the addition of phytase (500 U/Kg diet), citric acid (5% of diet) and) or both (phytase 1000 U/Kg and 5% citric acid). The results showed that the thermal process did not have a significant impact on the apparent digestibility of dry matter, crude protein and minerals. In low-ash diets, phytase supplementation increased the apparent digestibility of phosphorus, protein, ash, calcium, magnesium, copper, iron, strontium and zinc in low-ash diets containing soybean meal, but had little effect in high-ash diets. Finally, in low-ash diets the addition of citric acid increased the phytase effect while its addition in the high-ash diets had the opposite results. Cheng and Hardy, (2003) evaluated the effects of extrusion processing, expelling processing, and phytase supplementation in extruded full-fat soybeans and expelled soybeans, on apparent digestibility coefficients of dry matter, crude protein, amino acids, phosphorus and other minerals in rainbow trout feeds. Rainbow trout (170.8 g) were fed eight diets (30% incorporation into a casein-gelatin reference diet) consisting of raw full-fat soybeans which was used as a reference diet without supplementation of phytase, extruded soybeans with incremental addition of phytase (0, 200, 400, 600, 800 and 1000 FTU/Kg of diet) and expelled soybeans with addition of 200 FTU/Kg of diet. The results showed that extrusion process increased the ADC value of crude protein as well as that of all the

amino acids and sulfur but reduced magnesium and total-phosphorus ADC values compared to raw soybeans. On the other hand, increasing the phytase supplementation resulted in increased ADC values for total phosphorus, phytate-phosphorus, manganese and zinc. The optimal level of phytase supplementation was found to be 400 FTU/Kg of diet for rainbow trout fed diets containing full-fat soybeans. Cheng and Hardy (2004a) conducted a series of three experiments to evaluate the effects of microbial phytase and its dosage on apparent digestibility coefficients of dry matter, crude protein, amino acids, and minerals in soybean product-based diets for rainbow trout. Phytase of microbial origin was supplemented at incremental levels (0, 500, 1000, 2000 and 4000 FTU/Kg diet) in each one of the three diets including: two semi purified diets (soy protein concentrate-gelatin-dextrin and soybean meal-gelatin-dextrin) and a practical diet (soybean meal-fishmeal-wheat gluten based). The results with the two semi purified diets supplemented with the microbial phytase showed a significant increase in mineral digestibility, except for copper and iron. On the other hand, results of practical diet supplementation showed a significant increase of apparent digestibility coefficients of dry matter, crude protein, amino acids (except tryptophan and tyrosine), and minerals (except copper and iron). The authors concluded that the optimum dose of phytase supplementation in rainbow trout diets was 500 FTU/kg diet. Cheng and Hardy (2004b) conducted two experiments in order to evaluate the effects that phytase supplementation in corn distiller's dried grain with solubles (DDGS) had on apparent digestibility coefficients of nutrients, growth performance and apparent nutrient retention. In the first experiment, fish were fed DDGS supplemented with incremental levels of phytase (0, 300, 600, 900 and 1200 FTU/Kg diet). In the second experiment, rainbow trout were fed six diets consisting of a basal diet (containing 15% DDGS and supplemented with lysine and methionine) without and with supplementation of phytase (500 FTU/Kg diet) and incremental levels of trace mineral premix (0.02, 0.04, 0.06, 0.08 and 0.1%). The results from the first experiment showed that ADC of dry matter was positively affected only by supplementation with 300 FTU of phytase, ADC of crude fat was significantly higher in the 600 FTU phytase treatment, and ADC of crude protein was significantly higher for the 900 and 1200 FTU phytase treatments. Supplementation of phytase increased

ADC values significantly for all the amino acids, calcium, magnesium, manganese, zinc, total phosphorus and phytate-phosphorus. In the second experiment at the end of a ten-week feeding period, the results showed no effect of supplementation on growth performance, body composition, or survival. Regarding nutrient retention, only the fish fed the basal diet without trace mineral supplementation showed lower zinc and manganese levels. The authors concluded that when phytase is used in rainbow trout diets, trace mineral supplementation levels could be reduced. In a study conducted by Yang et al. (2011), soybean meal pretreated with phytase was used to replace fishmeal in graded levels (0, 20, 40, 60 and 80%) in the diet of juvenile rainbow trout (4 g) and the effects on growth performance were evaluated after 90 days of feeding. The results showed no statistically significant difference on growth parameters when fish were fed the diets up to 60% SBM inclusion. The phytase treated SBM diet also led to a decrease in total phosphorus excretion by the fish while an increase in nitrogen excretion was also reported. Dalsgaard et al. (2012) investigated if apparent nutrient digestibility values of rainbow trout (110 g) were improved by supplementing with three different enzyme-supplemented diets formulated with high inclusion levels of plant-based proteins. Three diets containing 34.4% soybean meal, 24.6% sunflower meal and 26.4% rapeseed meal were individually coated, post-extrusion, with either β-glucanase, xylanase, protease, or all the three combined. Enzyme supplementation did not significantly improve apparent nutrient digestibility of sunflower and rapeseed-based diets, but in contrast β-glucanase and protease significantly improved apparent digestibility of all dietary nutrients of the soybean-based diet.

As mentioned, the amino acid profiles of plant proteins differ from that of fishmeal, and numerous researchers have investigated the effects of supplementing amino acids in diets containing plant proteins to improve fish growth performance. Davies and Morris (1997) evaluated the effects of individual and multiple amino acid supplementation in diets where soybean meal was used as the principle protein source on the growth performance of rainbow trout. Rainbow trout juveniles (50 g) were fed for nine weeks either a control diet (fishmeal), or a soybean-based diet (fishmeal replaced at 66%). The soybean-based diet was then supplemented with

methionine, methionine and lysine, lysine, or methionine lysine, threonine, histidine, arginine and tryptophan. The results showed fishmeal replacement at 66% by soybean meal reduced the growth performance of rainbow trout while supplementing with methionine or methionine and lysine did not improve feed efficiency, apparent net protein utilization or weight gain. However, the multiple amino acid supplementation of the soybean-based diet led to a significant improvement in both weight gain and feed utilization but both parameters were still inferior to those of fish fed the control diet. Cheng et al. (2003), conducted a study to determine the effects of lysine supplementation in diets in which fishmeal was partially replaced by plant proteins on trout performance. Rainbow trout with an average weight of 14.9 g were fed seven diets consisting of a reference diet (32.1% fishmeal, 46% crude protein and 2.25% lysine), and a basal diet (15% fishmeal, 43% crude protein and 1.5% lysine) supplemented incrementally with lysine in order to reach 1.65, 1.8, 1.95, 2.1 and 2.25% lysine content. At the end of the eight-week trial, fish fed the plant protein-based diets supplemented with 0.4% or higher lysine did not differ statistically in growth performance compared to the reference fishmeal-based diet. Lysine supplementation in plant protein-based diets increased crude protein and lysine levels, and reduced fat content in the whole body of rainbow trout. However, feed conversion ratios were higher when lysine was supplemented, indicating less efficient nutrient retention. In an experiment conduct by Luo et al. (2006), rainbow trout (39.2 g) were fed six experimental diets containing solvent-extracted cottonseed meal at graded levels of 0 (fishmeal-based diet), 25, 50, 75 and 100% and a sixth diet which was based upon the formulation of the 75% cottonseed meal supplemented with lysine and methionine in order be similar to the 0% diet which was the fishmeal-based diet. Results showed fishmeal can be replaced by solvent-extracted cottonseed meal up to 50% in the diets of rainbow trout without negatively affecting growth and nutrient utilization.

A study conducted by Gaylord et al. (2009) to test the hypothesis that by balancing a plant-based diet on available amino acid basis to the profile of rainbow trout muscle could result in a reduction of dietary protein level and equal growth performance of rainbow trout (initial weight 20 g). The dietary treatments used were

the basal diets containing either 45% or 35% intact plant protein and the same basal diets supplemented with lysine, methionine, threonine and glycine in order to be equivalent to 45% crude protein from rainbow trout muscle on an available amino acid basis and a last diet formulated to be equal with the 35% diet and supplemented with all the previous mentioned amino acids but lacking glycine. At the end of the 12-week trial, the results showed that amino acid supplementation improved weight gain, protein retention efficiency and muscle ratio compared to the corresponding intact protein diet while glycine supplementation did not show any beneficial effect on fish performance. Alami-Durante at el. (2010) performed a study in order to determine how changes in dietary plant protein sources and amino acid profiles affected the muscle growth process of rainbow trout. A feeding trial was conducted for 12 weeks and juvenile rainbow trout with a 14 g initial body weight were fed two diets containing fish meal or a mixture of plant protein sources either low (2.5%) or high in soybean meal (33%). Both diets were supplemented with crystalline indispensable amino acids (IAA) to match the rainbow trout muscle IAA profile. At the end of the trial no statistically significant differences were detected for overall somatic growth and daily nitrogen gain although protein and feed efficiency were higher in favor of the control diet. Muscle growth in the study were affected by the treatments with the diet high in soybean meal leading to a significant decrease in mean and median diameter of muscle fibers. A significant decrease in the expression of MyoD and a significant increase in expression of fast-myosin heavy chain (MHC), were observed but no significant changes in myogenin expression was detected. Zhang et al. (2012) investigated if rainbow trout could utilize diets with >95% plant protein supplemented with essential amino acids. Their study was designed to define the optimal combinations of plant protein concentrates based on different criteria such as growth rate, feed conversion ratio and digestibility with the help of mixture models. Rainbow trout juveniles, initial weight 61 g, were fed eight diets consisting of a fishmeal-based reference diet, three diets which were mixtures of plant protein concentrates (P-MIX with 49% pea and 49% potato protein concentrates, C-MIX with 49% potato and 49% canola protein concentrates and S-MIX with 93% soy protein concentrate of diet) in which were added the two limiting amino acids and taurine, and four diets which were

combinations of the three previous diets (P-MIX+C-MIX, P-MIX+S-MIX, C-MIX+S-MIX and P-MIX+C-MIX+S-MIX). All the plant protein-based diets were supplemented with 5% krill products as an attractant. After a 72-day feeding trial, the results showed no significant differences in weight gain among the dietary treatments. Feed intake was higher for the C-MIX and S-MIX diets. A combination of P-MIX and C-MIX resulted in the most efficient feed conversion ratio. Retention of dietary N was highest when a combination of P-MIX and S-MIX was used, while retention of digestible N was highest for a combination of P-MIX and C-MIX.

Bodin et al. (2012) carried out two experiments with rainbow trout fry (0.70 g) and large juveniles (2.85 g). The two groups were fed to satiation (two times/day) for 9, 17 and 25 days, five different diets containing graded levels of coated amino acids (0, 25, 50. 75 and 100%) replacing fishmeal (cod muscle meal). At the end of the trials, the response variables evaluated were growth rate, protein retention, fat deposition and feed intake. The results showed that the molecular form of nitrogen in the diet affected growth rate, feed intake and protein deposition for diets containing more 50% of protein as coated amino acids. Finally, although the larger juveniles responded better to the treatment, the authors concluded that a period of 17 days was necessary for both sizes of fish to adapt to the diets with 65% inclusion of coated free amino acid to achieve 85% of the potential maximum growth rate. Wacyk et al. (2012) investigated the effect of increased supplementation of branched chain amino acids in either fishmeal-based or plant-based (soy protein isolate) diets on fish growth, nutrient utilization plasma variables and hepatic gene expression in rainbow trout juveniles (12 g initial weight). After a period of 12 weeks, the fish group fed the plant-based diet showed a significantly lower growth rate, higher feed intake, higher feed conversion ratio, lower nutrient retention and higher plasma total free amino acid concentration. Amino acid supplementation in the plant-based diet lowered the lipid content of the fish. Moreover, the results from hepatic gene expression showed mainly an effect of the plant-based diet on liver metabolism with reduced expression of alanine aminotransferase and glutamine synthetase while increasing expression of aspartate aminotransferase and asparagine synthetase compared to the fishmeal-based diet. Rolland et al. (2015) investigated the effects of dietary methionine supplementation in

plant-based diets on growth and hepatic expression of genes related to the somatotropic axis and protein turnover. Rainbow trout, initial average weight 116.8 g, were fed five diets consisting of a plant-based basal diet (wheat gluten, soya protein concentrate and pea protein concentrate) supplemented with DL methionine at levels of 0, 0.1, 0.3, 0.4 and 0.7% for six weeks. The results showed that increasing crystalline methionine levels in the diets of rainbow trout significantly improved growth, feed conversion and protein utilization. Moreover, the transcript levels of GHR-I and IGF-I increased linearly with an increase of dietary methionine content. In contrast, hepatic protein degradation, as measured by the expression of the following genes, Prot 20D, Capn 1, Capn 2, CAST-L and CAST-S, decreased with increasing dietary methionine level in a relatively linear manner.

2.5.1 Summary of Fishmeal Replacement Studies

In summary, the literature review on fishmeal replacement studies using rainbow trout as the experimental animal led to several findings. Even though, the major goal of these studies was to contribute eventually to replace fishmeal at the highest level possible, all of them, one way or another, demonstrated that there are unknown limiting factors impeding full fishmeal replacement. Replacing half of fishmeal protein in a diet with single plant proteins can be achieved without causing significant effects on fish performance. However, in most of the studies the remaining proportion of fishmeal in the diet is still much higher compared to current formulations adopted by the aquafeed industry. Higher replacement levels require supplementing diets with amino acids to meet minimum dietary requirements, but even with amino acid supplementation fish growth performance remains inferior compared to high fishmeal diets. Enzymatic treatment of plant origin ingredients shows improved nutrient utilization up to a limit, indicating there are other factors responsible for the observed adverse effects on fish physiology. Finally, the age of the fish and the experimental duration in many studies were found to be major limiting factors. The majority of the studies used juvenile fish and the experimental periods selected are considered short-term (9–12 weeks). These indices limit the true evaluation of the

impact that an alternative plant protein can have on fish physiology and zootechnical performances throughout an entire production cycle.

2.6 Plasma Amino Acids

In general, most of the fishmeal replacement studies have been based on growth and digestibility trials, even though these response variables provide little insight into specific rates of nutrient absorption and metabolism (Karlsson et al., 2006). Plasma free amino acid concentrations have been suggested as a tool for protein quality evaluation (Yun et al., 2015). Even though several studies have demonstrated differences in the uptake between dietary crystalline amino acids and amino acids derived from intact protein (Yamada et al., 1981; Murai et al., 1987: Schuhmacher et al., 1995; Schuhmacher et al., 1997), few studies have focused on monitoring the postprandial plasma amino acid concentrations in rainbow trout during fishmeal replacement. Walton and Wilson (1986) investigated post-prandial changes in plasma free amino acids in rainbow trout after feeding a complete diet containing casein as the protein source and found a positive correlation coefficient between dietary and plasma essential amino acid concentrations.

Yamamoto et al. (1998) conducted a study to investigate the effects of different practical protein sources (fishmeal, defatted soybean meal and malt protein flour) had on the plasma free amino acid concentrations in rainbow trout. The results showed that plasma amino acids in the soybean meal and malt protein flour groups peaked later than the fishmeal group. The authors proposed that the temporal differences observed in plasma free amino acids between the ingredients are due in part to different protein digestive processes in the gastrointestinal tract (Yamamoto et al., 1998). However, there are other proposed factors which could affect the rates of digestion including type of protein, other dietary ingredients, meal size, fish size and temperature (Walton and Wilson, 1986). Larsen et al. (2012) conducted a study with juvenile rainbow trout fed either a fishmeal-based diet or plant protein diet (59% of fishmeal protein replaced by wheat, peas, field beans, sunflower and soybean) and monitored postprandial plasma amino acid concentrations over time following a single meal. Free essential amino acid concentrations appeared less synchronized, broader

peaks and in general delayed in fish fed the plant protein diet compared to the fishmeal-based diet suggesting that not only the amino acid profile but also absorption kinetics affects protein accretion by determining the postprandial anabolic or catabolic fate of amino acids (Larsen et al., 2012). In order for an organism to efficiently utilize amino acids from different ingredients, amino acids derived from each ingredient should be present simultaneously in plasma with a balanced composition (Yamamoto et al., 1998). However, most of the studies have focused only on plasma samples collected from the caudal vein which only provides information about amino acid metabolism in the periphery and not regarding the rate of uptake and release by the liver after a meal (Murai et al., 1987). Karlsson et al. (2006) conducted a study to investigate the post-prandial amino acid uptake and immediate metabolic processing of amino acids in rainbow trout by simultaneously sampling blood from the hepatic portal vein (pre-hepatic) and the dorsal aorta (post-hepatic) using cannulated rainbow trout force-fed either a fishmeal-based meal or a fishmeal-corn gluten-based meal. The results showed that the total free amino acid concentrations were consistently higher in the hepatic portal vein than in the dorsal aorta, demonstrating the that plasma free amino acid measurements in the hepatic portal vein provide a much greater resolution of the uptake profiles than measurements from the systemic blood.

2.7 Genetic Selection

Genetic selection of trout to improve performance began in the early 1920s with efforts to select brook trout for increased resistance to furunculosis, caused by *Aeromonas salmonicida (*Embody and Hayford, 1925*)*. Another example of early trout selection involved development of the Donaldson strain of rainbow trout beginning in 1932 (Donaldson and Olson, 1957). After generations of selection based on phenotypic traits, a significant increase in growth and fecundity was reported (Hulata, 2001).

In 1971 selective breeding efforts with salmon and rainbow trout were initiated in Norway. For the first two generations of selection, the breeding goal was increased growth rate. Age at sexual maturation was then included as a selection trait with the aim of reducing precocious maturation that lowered the value of farmed fish

(Gjerdrem, 2000). During the fifth generation, disease resistance (against furunculosis and ISA virus) and meat quality were included as selection traits (Gjerdrem, 2000). By 2000, 65% of the salmon and trout produced in Norway were offspring of genetically improved fish (Gjerdrem, 2000). Until the early 1990s, a relatively small number of aquaculture breeding programs existed worldwide. Several large-scale selection experiments and breeding programs, aimed at increasing growth rate, were conducted resulting in 10–20% gain per generation in channel catfish, rainbow trout, Atlantic salmon, coho salmon, Nile tilapia and other tilapia species (Hulata, 2001).

A long-term selective breeding program was undertaken at the University of Idaho to improve performance of rainbow trout fed diets in which plant protein concentrates supplied the protein (Overturf et al., 2004). The selection criteria were relatively simple; offspring of family crosses were evaluated to identify individuals that that flourished on these diets and these were used to produce the next generation. Selection of rainbow trout families and individuals within families was based on weight gain and feed conversion ratio (Overturf et al., 2004). This approach yielded a gain in performance of 10-15% per generation. This selection program demonstrated that selective breeding is an effective complementary method to improve biological utilization of novel feed ingredients by farmed fish (Quinton et al., 2007). Breeding programs now exist for all major farmed carnivorous fish species, and have achieved improvements in growth, feed efficiency, disease resistance and product quality traits (Quinton et al., 2007).

While rainbow trout selective breeding programs have successfully enhanced phenotypic traits important to sustainability and economic viability of the trout aquaculture sector, there have not been many research studies that delved deeper into the subject to answer important genetic questions. For example, does selection for performance of fish fed a plant protein-based diet also confer improved performance when selected fish are fed a fishmeal-based diet or vice-versa? Palti et al. (2006) conducted a study with objective to evaluate familial growth response of a commercial strain of rainbow trout selected for improved growth on fishmeal-based diets when fed a plant-based diet (corn and wheat gluten-based) and assess the

magnitude of genotype x diet interactions. They found that even though the strain of rainbow trout was selected for improved growth on fishmeal-based diets, growth performance improvements were also found when the fish were fed plant protein-based diets.

Pierce et al. (2008) conducted a follow-up to that of Palti et al. (2006) to assess the possible genotype x interaction in a commercial rainbow trout when fed a plant-based diet using commercially relevant ingredients, i.e. corn gluten meal, soybean meal, with a low level of wheat gluten. Fish were fed to a final body weight of 600 g. The results showed substantial genetic variation for utilizing plant-based diets containing soybean meal in the commercial rainbow trout strain, indicating that selection of trout for growth on a fishmeal diet does not confer increased performance when the same fish are fed a plant-based diet.

Dupont-Nivet et al. examined the genetic variability and genotype x diet interactions during early growth of rainbow trout heterozygous clones fed either a fishmeal-based diet or a plant protein-based diet (white sweet lupin, corn gluten, wheat gluten, extruded dehulled peas and soybean meal) for a period of 49 days. The results showed that there are significant genotype x diet interactions when feeding juvenile rainbow trout with an all plant-protein diet indicating that a high performing genotype on a fishmeal diet may perform poorly when fed a plant-protein diet, confirming the observations of Pierce et al. (2008). Furthermore, it was noted that plant-based diets are likely to enhance overall phenotypic variance in a population, regardless of its genetic variability (Dupont-Nivet et al., 2009).

Le Boucher et al. (2011) tried to estimate accurate heritability and genotype x diet interactions for growth and quality traits of rainbow trout fed a plant-based diet (corn gluten, wheat gluten, soybean meal, white lupin and extruded dehulled pea) from first feeding. Because of the reduced growth of fish fed the plant-based diet, the estimation of genetic parameters was done on fish from both dietary treatments measured at the same age and also on fish measured at the same weight. The fish group fed the plant-based diet for 343 days weighed almost 50% less than the fishmeal-fed group (216 g vs 440 g). The main finding of the study was the high

heritability observed in fish fed the plant-based diet for body weight, TGC and the generally low, though significant, genotype x diet interactions for final body weight, fork length, carcass yield, viscera yield, head yield and filet yield between fish fed source-contrasted diets.

In a subsequent study Le Boucher et al. (2012) tried to show if rainbow trout can actually be selected for their ability to adapt to a diet totally free of marine ingredients and to characterize the effects of such a selection scheme on major production traits. The results showed that after a single generation of selection for the ability to adapt to a totally plant-based diet, mean body weight and biomass and survival can be improved. Also, at least two major production traits (survival and growth) associated with the ability to adapt to plant-based diet were positively modified after a single generation of selection, indicating that domesticated populations of rainbow trout have the genetic potential to adapt rapidly to major dietary changes. Callet et al. (2017) conducted a study to evaluate the overall growth performance of a selected rainbow trout strain fed an all plant-based diet for three generations when compare to a control line strain and fed either a fishmeal-based diet or an all plant-based diet for a period of 197 days. The selected strain fed the all plant-based diet grew at the same rate as the control line fed the fishmeal-based diet, However the enhanced performance on the all plant-based diet seems to be mostly linked to a higher feed intake for the selected fish. Regarding nutrient retention, no interaction effect or strain effect was detected. A noteworthy finding of the study was related to the apparent digestibility coefficients showing that there was no interaction effect detected. However, a strong diet effect in the fish fed the all plant-based diet showed a significant decrease of apparent digestibility coefficients of moisture, lipid, energy and starch and higher value for the protein.

Finally, Overturf et al. (2013) evaluated a strain of rainbow trout selected for four generations for improved growth when fed a fishmeal-free, plant-protein based diet and compared it with two of its parental strains (one fast growing domesticated strain and one slow growing conservation strain) on growth performances and nutrient retention when fed either a fishmeal-based diet or the selection plant protein-based

diet. At the end of the 12-week trial, the results showed a significant interaction for strain and diet, with the highest values for weight gain and specific growth rate being recorded among all treatments for the selected strain fed the plant protein-based diet. Regarding nutrient retention a significant interaction was found for protein retention efficiency with the selected strain also showing the highest values. Moreover, Venold et al. (2012) studying the same selected strain of rainbow trout found that this strain was less sensitive to intestinal inflammation induced by dietary soybean meal, thereby showing that selection for traits such as improved growth and nutrient utilization on a plant-protein based diet can lead to indirect selection for improved gut health.

These studies clearly demonstrate that selection of trout for weight gain yields positive gains within a few generations, and that selection for growth performance when fish are fed a plant protein-based diet also yields positive gains. However, gaps in knowledge remain. Research has not yet shown what physiological mechanisms are responsible for increased performance of selected fish. Even though apparent digestibility (ADC) of protein has been shown to be similar or even superior to fishmeal, results from replacement studies indicate reduced growth and nutrient utilization when trout are fed low fishmeal diets with plant proteins suppling the bulk of dietary protein. Although Callet et al. (2017) did not detect any interaction effect of strain by diet on ADC values after three generations of selection, it is unclear if the results of an improved growth and protein retention in the selected strain reported by Overturf et al. (2013) are the result of improved nutrient digestibility or lower protein turnover. Lower protein turnover in growing animals is often the result of a reduced protein degradation and hence high efficiencies of retention of synthesized proteins, which could be the case for the selected fish. Finally, selection for improved growth could conceivably lead to an accelerated digestive rate or another mechanism associated with the digestion process resulting in more synchronous dietary amino acid release and uptake by the fish. This remains a plausible hypothesis to explain improved fish growth performance associated with selection for performance when fed a high-soy, plant protein-based diet.

2.8 Bibliography

Ai, Y. and J. l. Jane. 2016. “Macronutrients in Corn and Human Nutrition.” *Comprehensive Reviews in Food Science and Food Safety* 15(3):581–98.

Alami-Durante, H., C. Wrutniak-Cabello, S. J. Kaushik, and F. Médale. 2010. “Skeletal Muscle Cellularity and Expression of Myogenic Regulatory Factors and Myosin Heavy Chains in Rainbow Trout (*Oncorhynchus mykiss*): Effects of Changes in Dietary Plant Protein Sources and Amino Acid Profiles.” *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 156(4):561–68.

Allan, G. L. 2000. “Potential for Pulses in Aquaculture Systems.” Pp. 507–16 in. Springer, Dordrecht.

Appelqvist, H., P. Wäster, K. Kågedal, and K. Öllinger. 2013. “The Lysosome: From Waste Bag to Potential Therapeutic Target.” *Journal of Molecular Cell Biology* 5(4):214–26.

Apper-Bossard, E., A. Feneuil, A. Wagner, and F. Respondek. 2013. “Use of Vital Wheat Gluten in Aquaculture Feeds.” *Aquatic Biosystems* 9(1).

Arntfield, S. D. 2004. “Proteins from Oil-Producing Plants.” *Proteins in Food Processing* 146–75.

Awika, J. M. 2011. “Major Cereal Grains Production and Use around the World.” Pp. 1–13 in.

Baker, D. H. 2008. “Animal Models in Nutrition Research.” *The Journal of Nutrition* 138(2):391–96.

Bakke, A., C. Glover, and Å. Krogdahl. 2010. “Feeding, Digestion and Absorption of Nutrients.” *Fish Physiology* 30:57–110.

Barrows, F. T., T. G. Gaylord, D. A. J. Stone, and C. E. Smith. 2007. “Effect of Protein Source and Nutrient Density on Growth Efficiency, Histology and Plasma Amino

Acid Concentration of Rainbow Trout (*Oncorhynchus mykiss* Walbaum)." *Aquaculture Research* 38(16):1747–58.

Bodin, N., G. Delfosse, T. T. N. Thu, E. Le Boulengé, T. Abboudi, Y. Larondelle, and X. Rollin. 2012. "Effects of Fish Size and Diet Adaptation on Growth Performances and Nitrogen Utilization of Rainbow Trout (*Oncorhynchus mykiss* W.) Juveniles given Diets Based on Free and/or Protein-Bound Amino Acids." *Aquaculture* 356–357:105–15.

Le Boucher, R., M. Dupont-Nivet, M. Vandeputte, T. Kerneïs, L. Goardon, L. Labbé, B. Chatain, M. J. Bothaire, L. Larroquet, F. Médale, and E. Quillet. 2012. "Selection for Adaptation to Dietary Shifts: Towards Sustainable Breeding of Carnivorous Fish." *PLoS ONE* 7(9):3–9.

Le Boucher, R., E. Quillet, M. Vandeputte, J. M. Lecalvez, L. Goardon, B. Chatain, F. Médale, and M. Dupont-Nivet. 2011. "Plant-Based Diet in Rainbow Trout (*Oncorhynchus mykiss* Walbaum): Are There Genotype-Diet Interactions for Main Production Traits When Fish Are Fed Marine vs. Plant-Based Diets from the First Meal?" *Aquaculture* 321(1–2):41–48.

Bröer, S. 2008. "Amino Acid Transport Across Mammalian Intestinal and Renal Epithelia." *Physiological Reviews* 88(1):249–86.

Bureau, D. P. and P. M. Encarnação. 2006. "Adequately Defining the Amino Acid Requirements of Fish: The Case Example of Lysine." *Simposium Internacional De Nutrición Acuícola; Avances En Nutrición Acuícola* 8:29–54.

Burr, G. S., W. R. Wolters, F. T. Barrows, and R. W. Hardy. 2012. "Replacing Fishmeal with Blends of Alternative Proteins on Growth Performance of Rainbow Trout (*Oncorhynchus mykiss*), and Early or Late Stage Juvenile Atlantic Salmon (*Salmo salar*)." *Aquaculture* 334–337:110–16.

Calles, T. 2016. "Preface to Special Issue on Leguminous Pulses." *Plant Cell, Tissue and Organ Culture (PCTOC)* 127(3):541–42.

Callet, T., F. Médale, L. Larroquet, A. Surget, P. Aguirre, T. Kerneis, L. Labbé, E. Quillet, I. Geurden, S. Skiba-Cassy, and M. Dupont-Nivet. 2017. “Successful Selection of Rainbow Trout (*Oncorhynchus mykiss*) on Their Ability to Grow with a Diet Completely Devoid of Fishmeal and Fish Oil, and Correlated Changes in Nutritional Traits.” *PLoS ONE* 12(10):1–21.

Carrier, D. J., S. Ramaswamy, and C. Bergeron. 2012. *Biorefinery Co-Products : Phytochemicals, Primary Metabolites and Value-Added Biomass Processing*. John Wiley & Sons.

Chen, L., K. Wang, A. Long, L. Jia, Y. Zhang, H. Deng, Y. Li, J. Han, and Y. Wang. 2017. “Fasting-Induced Hormonal Regulation of Lysosomal Function.” *Cell Research* 27(6):748–63.

Cheng, Z. J. and R. W. Hardy. 2003. “Effects of Extrusion Processing of Feed Ingredients on Apparent Digestibility Coefficients of Nutrients for Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture Nutrition* 9(2):77–83.

Cheng, Z. J. and R. W. Hardy. 2004. “Effects of Microbial Phytase Supplementation in Corn Distiller’s Dried Grain with Solubles on Nutrient Digestibility and Growth Performance of Rainbow Trout, *Oncorhynchus mykiss*.” *Journal of Applied Aquaculture* 15(3–4):83–100.

Cheng, Z. J., R. W. Hardy, and J. L. Usry. 2003. “Effects of Lysine Supplementation in Plant Protein-Based Diets on the Performance of Rainbow Trout (*Oncorhynchus mykiss*) and Apparent Digestibility Coefficients of Nutrients.” *Aquaculture* 215(1–4):255–65.

Cheng, Z. J., R. W. Hardy, V. Verlhac, and J. Gabaudan. 2004. “Effects of Microbial Phytase Supplementation and Dosage on Apparent Digestibility Coefficients of Nutrients and Dry Matter in Soybean Product-Based Diets for Rainbow Trout *Oncorhynchus mykiss*.” *Journal of the World Aquaculture Society* 35(1):1–15.

Collins, S. A., A. R. Desai, G. S. Mansfield, J. E. Hill, A. G. Van Kessel, and M. D. Drew. 2012. “The Effect of Increasing Inclusion Rates of Soybean, Pea and

Canola Meals and Their Protein Concentrates on the Growth of Rainbow Trout: Concepts in Diet Formulation and Experimental Design for Ingredient Evaluation." *Aquaculture* 344–349:90–99.

Cowey, C. B. 1995. "Protein and Amino Acid Requirements: A Critique of Methods." *Journal of Applied Ichthyology* 11(3–4):199–204.

Dabrowski, K. 1986. "Protein Digestion and Amino Acid Absorption along the Intestine of the Common Carp (*Cyprinus carpio L*.), a Stomachless Fish: An in Vivo Study." *Reproduction, Nutrition, Developpement* 26(3):755–66.

Dabrowski, K. and H. Guderley. 2003. "Intermediary Metabolism." *Fish Nutrition* 309–65.

Dai, Z. L., X. L. Li, P. B. Xi, J. Zhang, G. Wu, and W. Y. Zhu. 2012. "Metabolism of Select Amino Acids in Bacteria from the Pig Small Intestine." *Amino Acids* 42(5):1597–1608.

Dalsgaard, J., V. Verlhac, N. H. Hjermitslev, K. S. Ekmann, M. Fischer, M. Klausen, and P. B. Pedersen. 2012. "Effects of Exogenous Enzymes on Apparent Nutrient Digestibility in Rainbow Trout (*Oncorhynchus mykiss*) Fed Diets with High Inclusion of Plant-Based Protein." *Animal Feed Science and Technology* 171(2–4):181–91.

Davies, S. J. and P. C. Morris. 1997. "Influence of Multiple Amino Acid Supplementation on the Performance of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), Fed Soya Based Diets." *Aquaculture Research* 28(1):65–74.

Day, O. J. and H. G. Plascencia GonzAlez. 2000. "Soybean Protein Concentrate as a Protein Source for Turbot *Scophthalmus maximus L*." *Aquaculture Nutrition* 6(4):221–28.

DeLong, D. C., J. E. Halver, and E. T. Mertz. 1958. "Nutrition of Salmonoid Fishes." *The Journal of Nutrition* 65(4):589–99.

Donaldson, L. R. and P. R. Olson. 1957. “Development of Rainbow Trout Brood Stock by Selective Breeding.” *Transactions of the American Fisheries Society* 85(1):93-101

Dupont-Nivet, M., F. Médale, J. Leonard, S. Le Guillou, F.Tiquet, E. Quillet, and I. Geurden. 2009. “Evidence of Genotype-Diet Interactions in the Response of Rainbow Trout (*Oncorhynchus mykiss*) Clones to a Diet with or without Fishmeal at Early Growth.” *Aquaculture* 295(1–2):15–21.

Embody, G. C. and C. O. Hayford. 2011. “The Advantage of Rearing Brook Trout Fingerlings from Selected Breeders.” *Transactions of the American Fisheries Society* 55(1):135-148

Enami, H. R. 2011. “A Review of Using Canola/Rapeseed Meal in Aquaculture Feeding.” *Journal of Fisheries and Aquatic Science* 6(1):22–36.

Escaffre, A., S. Kaushik, and M. Mambrini. 2007. “Morphometric Evaluation of Changes in the Digestive Tract of Rainbow Trout (*Oncorhynchus mykiss*) Due to Fish Meal Replacement with Soy Protein Concentrate.” *Aquaculture* 273(1):127–38.

Fauconneau, B. and M. Arnal. 1985. “In Vivo Protein Synthesis in Different Tissues and the Whole Body of Rainbow Trout (*Salmo gairdnerii R*.). Influence of Environmental Temperature.” *Comparative Biochemistry and Physiology. A, Comparative Physiology* 82(1):179–87.

de Francesco, M., G. Parisi, F. Médale, P. Lupi, S. J. Kaushik, and B. M. Poli. 2004. “Effect of Long-Term Feeding with a Plant Protein Mixture Based Diet on Growth and Body/Fillet Quality Traits of Large Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 236(1–4):413–29.

Francis, G., H. P. S. Makkar, and K. Becker. 2001. *Antinutritional Factors Present in Plant-Derived Alternate Fish Feed Ingredients and Their Effects in Fish*. Vol. 199.

Fraser, K. P. P. and A. D. Rogers. 2007. “Protein Metabolism in Marine Animals: The Underlying Mechanism of Growth.” Pp. 267–362 in *Advances in marine biology*. Vol. 52.

Freer, M. and H. Dove. 2002. *Sheep Nutrition*. CABI Pub. in association with CSIRO Pub.

Furuya, W. M., L. E. Pezzato, M. M. Barros, A. C. Pezzato, V. R. B. Furuya, and E. C. Miranda. 2004. “Use of Ideal Protein Concept for Precision Formulation of Amino Acid Levels in Fish-Meal-Free Diets for Juvenile Nile Tilapia (*Oreochromis niloticus L*.).” *Aquaculture Research* 35(12):1110–16.

Gatlin, D. M., F. T. Barrows, P. Brown, K. Dabrowski, T. G. Gaylord, R. W. Hardy, E. Herman, G. Hu, Å. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E. J. Souza, D. Stone, R. Wilson, and E. Wurtele. 2007. “Expanding the Utilization of Sustainable Plant Products in Aquafeeds: A Review.” *Aquaculture Research* 38(6):551–79.

Gaylord, T. G., and F. T. Barrows. 2009. “Multiple Amino Acid Supplementations to Reduce Dietary Protein in Plant-Based Rainbow Trout, *Oncorhynchus mykiss*, Feeds.” *Aquaculture* 287(1–2):180–84.

Gjedrem, T. 2000. “Genetic Improvement of Cold-Water ®sh Species.” 25–33.

Halver, J. E. 1957. “Nutrition of Salmonoid Fishes.” *The Journal of Nutrition* 62(2):225–43.

Halver, J. E. and W. E. Shanks. 1960. “Nutrition of Salmonoid Fishes.” *The Journal of Nutrition* 72(3):340–46.

Hardy, R. W. 1996. “Alternate Protein Sources for Salmon and Trout Diets.” *Animal Feed Science and Technology* 59(1–3):71–80.

Hardy, R. W. 2010. “Utilization of Plant Proteins in Fish Diets: Effects of Global Demand and Supplies of Fishmeal.” *Aquaculture Research* 41(5):770–76.

Hinkson, I. V. and J. E. Elias. 2011. “The Dynamic State of Protein Turnover: It’s about Time.” *Trends in Cell Biology* 21(5):293–303.

Houlihan, D. F., C. G. Carter, and I. D. McCarthy. 1995. Protein synthesis in fish. Pages 191–219 in Biochemistry and Molecular Biology of Fishes. Vol. 4. P. Hochachka and P. Mommsen, ed. Elsevier Science, Amsterdam, The Netherlands.

Hulata, G. 2001. “Genetic Manipulations in Aquaculture: A Review of Stock Improvement by Classical and Modern Technologies.” *Genetica* 111(1–3):155–73.

Hyde, R., P. M. Taylor, and H. S. Hundal. 2003. “Amino Acid Transporters: Roles in Amino Acid Sensing and Signalling in Animal Cells.” *The Biochemical Journal* 373(Pt 1):1–18.

Jahan-Mihan, A., B. L. Luhovyy, D. El Khoury, and G. H. Anderson. 2011. “Dietary Proteins as Determinants of Metabolic and Physiologic Functions of the Gastrointestinal Tract.” *Nutrients* 3(5):574–603.

Jiang, H. B., L. Q. Chen, and J. G. Qin. 2018. “Fishmeal Replacement by Soybean, Rapeseed and Cottonseed Meals in Hybrid Sturgeon *Acipenser baerii* ♀ × *Acipenser schrenckii* ♂.” *Aquaculture Nutrition* 24(4):1369–77.

Jürss, K. and R. Bastrop. 1995. “Chapter 7 Amino Acid Metabolism in Fish.” *Biochemistry and Molecular Biology of Fishes* 4:159–89.

Karlsson, A., E. J. Eliason, L. T. Mydland, A. P. Farrell, and A. Kiessling. 2006. “Postprandial Changes in Plasma Free Amino Acid Levels Obtained Simultaneously from the Hepatic Portal Vein and the Dorsal Aorta in Rainbow Trout (*Oncorhynchus mykiss*).” *Journal of Experimental Biology* 209(24):4885–94.

Kaushik, S. J., J. P. Cravedi, J. P. Lalles, J. Sumpter, B. Fauconneau, and M. Laroche. 1995. “Partial or Total Replacement of Fish Meal by Soybean Protein on Growth,

Protein Utilization, Potential Estrogenic or Antigenic Effects, Cholesterolemia and Flesh Quality in Rainbow Trout, *Oncorhynchus mykiss*." *Aquaculture* 133(3–4):257–74.

Kaushik, S. J. and P. Luquet. 1979. "Influence of Dietary Amino Acid Patterns on the Free Amino Acid Contents of Blood and Muscle of Rainbow Trout (*Salmo gairdnerii R.*)." *Comparative Biochemistry and Physiology. B, Comparative Biochemistry* 64(2):175–80.

Lall S. P. and Anderson S. 2005. "Amino Acid Nutrition of Salmonids: Dietary Requirements and Bioavailablity." *Cahiers Options Méditerranéennes* 63(May):1–90.

Larsen, B. K., J. Dalsgaard, and P. B. Pedersen. 2012. "Effects of Plant Proteins on Postprandial, Free Plasma Amino Acid Concentrations in Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture* 326–329:90–98.

Li, M. H. and E. H. Robinson. 2006. "Use of Cottonseed Meal in Aquatic Animal Diets: A Review." *North American Journal of Aquaculture* 68(1):14–22.

Little, K. L., B. M. Bohrer, H. H. Stein, and D. D. Boler. 2015. "Effects of Feeding High Protein or Conventional Canola Meal on Dry Cured and Conventionally Cured Bacon." *Meat Science* 103:28–38.

Lovell, T. 1998. "Dietary Requirements." Pp. 13–70 in *Nutrition and Feeding of Fish*. Boston, MA: Springer US.

Luo, L., M. Xue, X. Wu, X. Cai, H. Cao, and Y. Liang. 2006. "Partial or Total Replacement of Fishmeal by Solvent-Extracted Cottonseed Meal in Diets for Juvenile Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture Nutrition* 12(6):418–24.

Martínez Force, E., N. T. Dunford, and J. J. Salas. 2015. *Sunflower : Chemistry, Production, Processing, and Utilization*.

McKevith, B. 2005. "Nutritional Aspects of Oilseeds." *Nutrition Bulletin* 30(1):13–26.

Murai, T., Ogata, H., Hirasawa, Y., Akiyama, T. & Nose, T. 1987. Portal absorption and hepatic uptake of amino acids in rainbow trout force-fed diets containing casein or crystalline amino acids. *Nippon Suisan Gakkaishi*, 53, 1847–1859.

van Milgen, J. and J. Dourmad. 2015. “Concept and Application of Ideal Protein for Pigs.” *Journal of Animal Science and Biotechnology* 6(1):15.

Nagel, F., H. Slawski, H. Adem, R. P. Tressel, K. Wysujack, and C. Schulz. 2012. “Albumin and Globulin Rapeseed Protein Fractions as Fish Meal Alternative in Diets Fed to Rainbow Trout (*Oncorhynchus mykiss W*.).” *Aquaculture* 354–355:121–27.

Nakamura, S. and T. Yoshimori. 2017. “New Insights into Autophagosome–lysosome Fusion.” *J Cell Sci* 130(7):1209–16.

National Research Council (NRC). 2011. Nutrient Requirements of Fish and Shrimp. *National Academic Press*, Washington, DC.

Øverland, M., M. Sørensen, T. Storebakken, M. Penn, Å. Krogdahl, and A. Skrede. 2009. “Pea Protein Concentrate Substituting Fish Meal or Soybean Meal in Diets for Atlantic Salmon (*Salmo salar*) — Effect on Growth Performance, Nutrient Digestibility, Carcass Composition, Gut Health, and Physical Feed Quality.” *Aquaculture* 288(3–4):305–11.

Overturf, K., F. T. Barrows, and R. W. Hardy. 2013. “Effect and Interaction of Rainbow Trout Strain (*Oncorhynchus mykiss*) and Diet Type on Growth and Nutrient Retention.” *Aquaculture Research* 44(4):604–11.

Overturf, K., D. Bullock, S.t LaPatra, and R. Hardy. 2004. “Genetic Selection and Molecular Analysis of Domesticated Rainbow Trout for Enhanced Growth on Alternative Diet Sources.” *Environmental Biology of Fishes* 69(1–4):409–18.

Palmegiano, G. B., F. Daprà, G. Forneris, F. Gai, L. Gasco, K. Guo, P. G. Peiretti, B. Sicuro, and I. Zoccarato. 2006. “Rice Protein Concentrate Meal as a Potential

Ingredient in Practical Diets for Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture* 258(1–4):357–67.

Palti, Y., J. T. Silverstein, H. Wieman, J. G. Phillips, F. T. Barrows, and J. E. Parsons. 2006. "Evaluation of Family Growth Response to Fishmeal and Gluten-Based Diets in Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture* 255(1–4):548–56.

Panserat, S., C. Kolditz, N. Richard, E. Plagnes-Juan, F. Piumi, D. Esquerré, F. Médale, G. Corraze, and S. Kaushik. 2008. "Hepatic Gene Expression Profiles in Juvenile Rainbow Trout (*Oncorhynchus mykiss*) Fed Fishmeal or Fish Oil-Free Diets." *British Journal of Nutrition* 100(5):953–67.

Peters, J. M. 1994. "Proteasomes: Protein Degradation Machines of the Cell." *Trends in Biochemical Sciences* 19(9):377–82.

Pierce, L. R., Y. Palti, J. T. Silverstein, F. T. Barrows, E. M. Hallerman, and J. E. Parsons. 2008. "Family Growth Response to Fishmeal and Plant-Based Diets Shows Genotype × Diet Interaction in Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture* 278(1–4):37–42.

Písaříková, B. and Z. Zralý. 2009. "Nutritional Value of Lupine in the Diets for Pigs (a Review)." *Acta Veterinaria Brno* 78(3):399–409.

Pizauro, J. M., J. A. Ferro, A. C. F. de Lima, K. S. Routman, and M. C. Portella. 2004. "The Zymogen-Enteropeptidase System: A Practical Approach to Study the Regulation of Enzyme Activity by Proteolytic Cleavage." *Biochemistry and Molecular Biology Education* 32(1):45–48.

Quinton, C. D., A. Kause, J. Koskela, and O. Ritola. 2007. "Breeding Salmonids for Feed Efficiency in Current Fishmeal and Future Plant-Based Diet Environments." *Genetics Selection Evolution* 39(4):431–46.

Refstie, S., O. J. Korsoen, T. Storebakken, G. Baeverfjord, I. Lein, and a. J. Roem. 2000. "Differing Nutritional Responses to Dietary Soybean Meal in Rainbow Trout

(*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*).” *Aquaculture* 190(1–2):49–63.

Rolland, M., J. Dalsgaard, J. Holm, P. Gómez-Requeni, and P. V. Skov. 2015. “Dietary Methionine Level Affects Growth Performance and Hepatic Gene Expression of GH-IGF System and Protein Turnover Regulators in Rainbow Trout (*Oncorhynchus mykiss*) Fed Plant Protein-Based Diets.” *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* 181:33–41.

Romarheim, O. H., A. Skrede, Y. Gao, Å. Krogdahl, V. Denstadli, E. Lilleeng, and T. Storebakken. 2006. “Comparison of White Flakes and Toasted Soybean Meal Partly Replacing Fish Meal as Protein Source in Extruded Feed for Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 256(1–4):354–64.

Rust, M. B. 2003. “Nutritional Physiology.” *Fish Nutrition* 367–452.

Sanz, A., A. E. Morales, M. de la Higuera, and G. Gardenete. 1994. “Sunflower Meal Compared with Soybean Meals as Partial Substitutes for Fish Meal in Rainbow Trout (*Oncorhynchus mykiss*) Diets: Protein and Energy Utilization.” *Aquaculture* 128(3–4):287–300.

Schuhmacher, A., C. Wax, and J. M. Gropp. 1997. “Plasma Amino Acids in Rainbow Trout (*Oncorhynchus mykiss*) Fed Intact Protein or a Crystalline Amino Acid Diet.” *Aquaculture* 151(1–4):15–28.

Schuhmacher, A., J. Schön, M. Goldberg, and J. M. Gropp. 1995. “Plasma Amino Acid Levels in Rainbow Trout (*Oncorhynchus mykiss*).” *Journal of Applied Ichthyology* 11(3–4):309–16.

Seiliez, I., K. Dias, and B. M. Cleveland. 2014. “Contribution of the Autophagy-Lysosomal and Ubiquitin-Proteasomal Proteolytic Systems to Total Proteolysis in Rainbow Trout (*Oncorhynchus mykiss*) Myotubes.” *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 307(11):R1330–37.

Seiliez, I., J. Gabillard, M. Riflade, B. Sadoul, K. Dias, J. Avérous, S. Tesseraud, S. Skiba, and S. Panserat. 2012. “Amino Acids Downregulate the Expression of Several Autophagy-Related Genes in Rainbow Trout Myoblasts.” *Autophagy* 8(3):364–75.

Shanks, W. E., G. D. Gahimer, and J. E. Halver. 1962. “The Indispensable Amino Acids for Rainbow Trout.” *The Progressive Fish-Culturist* 24(2):68–73.

Sherasia, P. L., M. R. Garg, and B. M. Bhanderi. 2017. “Pulses and Their By-Products as Animal Feed.” *Pulses and Their By-Products as Animal Feed.*

Shewry, P. R. and N. G. Halford. 2002. “Cereal Seed Storage Proteins: Structures, Properties and Role in Grain Utilization.” *Journal of Experimental Botany* 53(370):947–58.

Skinner, M. M., B. K. Cross, and B. C. Moore. 2017. “Estimating *in Situ* Isotopic Turnover in Rainbow Trout (*Oncorhynchus mykiss*) Muscle and Liver Tissue.” *Journal of Freshwater Ecology* 32(1):209–17.

Slawski, H., H. Adem, R. P. Tressel, K. Wysujack, U. Koops, Y. Kotzamanis, S. Wuertz, and C. Schulz. 2012. “Total Fish Meal Replacement with Rapeseed Protein Concentrate in Diets Fed to Rainbow Trout (*Oncorhynchus mykiss Walbaum*).” *Aquaculture International* 20(3):443–53.

Small, B. C. and J. H. Soares. 1998. “Estimating the Quantitative Essential Amino Acid Requirements of Striped Bass Morone Saxatilis, Using Fillet A/E Ratios.” *Aquaculture Nutrition* 4(4):225–32.

Stein, H. H., L. L. Berger, J. K. Drackley, G. C. Fahey, D. C. Hernot, and C. M. Parsons. 2008. “Nutritional Properties and Feeding Values of Soybeans and Their Coproducts.” *Soybeans* 613–60.

Stone, D. A. J. 2003. “Dietary Carbohydrate Utilization by Fish.” *Reviews in Fisheries Science* 11(4):337–69.

Sugiura, S. H., J. Gabaudan, F. M. Dong, and R. W. Hardy. 2001. “Dietary Microbial Phytase Supplementation and the Utilization of Phosphorus, Trace Minerals and

Protein by Rainbow Trout [*Oncorhynchus mykiss* (Walbaum)] Fed Soybean Meal-Based Diets.” *Aquaculture Research* 32(7):583–92.

Sundell, K. S. and I. Rønnestad. 2011. “INTEGRATED FUNCTION AND CONTROL OF THE GUT | Intestinal Absorption.” *Encyclopedia of Fish Physiology* 1311–21.

Teskeredžić, Z., D. A. Higgs, B. S. Dosanjh, J. R. McBride, R. W. Hardy, R. M. Beames, J. D. Jones, M. Simell, T. Vaara, and R. B. Bridges. 1995. “Assessment of Undephytinized and Dephytinized Rapeseed Protein Concentrate as Sources of Dietary Protein for Juvenile Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 131(3–4):261–77.

Thiessen, D. L., G. L. Campbell, and P. D. Adelizi. 2003. “Digestibility and Growth Performance of Juvenile Rainbow Trout (*Oncorhynchus mykiss*) Fed with Pea and Canola Products.” *Aquaculture Nutrition* 9(2):67–75.

Thiessen, D. L., D. D. Maenz, R. W. Newkirk, H. L. Classen, and M. D. Drew. 2004. “Replacement of Fishmeal by Canola Protein Concentrate in Diets Fed to Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture Nutrition* 10(6):379–88.

Venold, F. F., M. H. Penn, Å. Krogdahl, and K. Overturf. 2012. “Severity of Soybean Meal Induced Distal Intestinal Inflammation, Enterocyte Proliferation Rate, and Fatty Acid Binding Protein (Fabp2) Level Differ between Strains of Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 364–365:281–92.

Verri, T., G. Terova, K. Dabrowski, and M. Saroglia. 2011. “Peptide Transport and Animal Growth: The Fish Paradigm.” *Biology Letters* 7(4):597–600.

Vilhelmsson, O. T., S. A. M. Martin, F. Médale, S. J. Kaushik, and D. F. Houlihan. 2004. “Dietary Plant-Protein Substitution Affects Hepatic Metabolism in Rainbow Trout (*Oncorhynchus mykiss*).” *British Journal of Nutrition* 92(01):71.

Wacyk, J., M. Powell, K. Rodnick, K. Overturf, R. A. Hill, and R. Hardy. 2012. “Dietary Protein Source Significantly Alters Growth Performance, Plasma Variables and Hepatic Gene Expression in Rainbow Trout (*Oncorhynchus mykiss*) Fed Amino Acid Balanced Diets.” *Aquaculture* 356–357:223–34.

Walton, M. J., R. M. Coloso, C. B. Cowey, J. W. Adron, and D. Knox. 1984. “The Effects of Dietary Tryptophan Levels on Growth and Metabolism of Rainbow Trout (*Salmo gairdneri*).” *The British Journal of Nutrition* 51(2):279–87.

Walton, M. J. and R. P. Wilson. 1986. “Postprandial Changes in Plasma and Liver Free Amino Acids of Rainbow Trout Fed Complete Diets Containing Casein.” *Aquaculture* 51(2):105–15.

Wilson, R.P. and Halver, J. E. 1986. “Protein and Amino Acid Requirements of Fishes.” *Annu. Rev. Nutr.* 6:225–44.

Wu, G. 1998. “Intestinal Mucosal Amino Acid Catabolism.” *The Journal of Nutrition* 128(8):1249–52.

Wu, G. 2009. “Amino Acids: Metabolism, Functions, and Nutrition.” *Amino Acids* 37(1):1–17.

Wu, G. 2013. *Amino Acids : Biochemistry and Nutrition*. *CRC Press.*

Xie, S. and A. Jokumsen. 1997. “Replacement of Fish Meal by Potato Protein Concentrate in Diets for Rainbow Trout, *Oncorhynchus mykiss* (Walbaum): Growth, Feed Utilization and Body Composition.” *Aquaculture Nutrition* 3(1):65–69.

Yaklich, R. W. 2001. “Beta-Conglycinin and Glycinin in High-Protein Soybean Seeds.” *Journal of Agricultural and Food Chemistry* 49(2):729–35.

Yamada, S., Simpson, K.L., Tanaka, Y., Katayama, T., 1981. Plasma amino acid changes in rainbow trout *Salmo gairdneri* force-fed casein and a corresponding amino acid mixture. *Bull. Jap. Soc. Sci. Fish.* 47, 1035–1040.

Yamamoto, T., Unuma, T., Akiyama, T. 1998. Postprandial changes in plasma free amino acid concentrations of rainbow trout fed diets containing different protein sources. Fish. Sci. 64, 474–481.

Yamamoto, T., Unuma, T. and Akiyama, T. 2000. “The Influence of Dietary Protein and Fat Levels on Tissue Free Amino Acid Levels of Fingerling Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 182(3–4):353–72.

Yang, Y. H., Y. Y. Wang, Y. Lu, and Q. Z. Li. 2011. “Effect of Replacing Fish Meal with Soybean Meal on Growth, Feed Utilization and Nitrogen and Phosphorus Excretion on Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture International* 19(3):405–19.

Yiğit, M., M. Bulut, S. Ergün, D. Güroy, M. Karga, O. S. Kesbiç, S. Yılmaz, Ü. Acar, and B. Güroy. 2012. *Journal of FisheriesSciences.Com.* Www.FisheriesSciences.com.

Yun, H., G. Park, I. Ok, K. Katya, S. Hung, and S. C. Bai. 2015. “Evaluation of Optimum Dietary Threonine Requirement by Plasma Free Threonine and Ammonia Concentrations in Surgically Modified Rainbow Trout, *Oncorhynchus mykiss*.” *Asian-Australasian Journal of Animal Sciences* 28(4):551–58.

Zhang, L., N. G. Gurskaya, E. M. Merzlyak, D.B. Staroverov, N. N. Mudrik, O. N. Samarkina, L. M. Vinokurov, S. Lukyanov, and K. A. Lukyanov. 2007. “Method for Real-Time Monitoring of Protein Degradation at the Single Cell Level.” *BioTechniques* 42(4):446–50.

Zhang, Y., M. Øverland, K. D. Shearer, M. Sørensen, L. T. Mydland, and T. Storebakken. 2012. “Optimizing Plant Protein Combinations in Fish Meal-Free Diets for Rainbow Trout (*Oncorhynchus mykiss*) by a Mixture Model.” *Aquaculture* 360–361:25–36.

Zhao, J., X. Zhang, H. Liu, M. A. Brown, and S. Qiao. 2018. “Dietary Protein and Gut Microbiota Composition and Function.” *Current Protein & Peptide Science* 20(2):145–54.

Zhao, L., S. M. Budge, A. E Ghaly, M. S. Brooks, and D. Dave. 2011. “Extraction, Purification and Characterization of Fish Pepsin: A Critical Review.” Journal of Food Processing & Technology 02(06):1–14.

Table 2.1. Salmon requirement for indispensable amino acids (percentage of diets containing 40 percent protein)

Amino Acid	Chinook	Coho
L-Arginine	2.4	2.4
L-Histidine	0.7	0.7
L-Isoleucine	0.9	
L-Leucine	1.6	
L-Lysine	2.0	1.5
L-Methionine[a]	0.5	0.5
L-Phenylalanine[b]	2.1	
L-Thereonine	0.9	
L-Tryptophan	0.2	0.2
L-Valine	1.3	

[a] In presence of 1% Cystine.
[b] In absence of Tyrosine.

CHAPTER 3

IMPROVED PERFORMANCE OF A RAINBOW TROUT SELECTED STRAIN IS ASSOCIATED WITH PROTEIN DIGESTION RATES AND SYNCHRONIZATION OF AMINO ACID ABSORPTION

3.1 Introduction

Replacement of fishmeal as the major protein source in feeds is critical for continued growth of the aquaculture industry as well as development of sustainable aquaculture (Naylor et al., 2009; Hardy, 2010; NRC 2011). Plant protein concentrates produced from grains, oilseeds and pulses are the leading alternative protein sources to replace fishmeal in fish feeds. However, numerous studies have shown suboptimal fish growth performance and reduced protein retention efficiency when carnivorous fish species are fed low-fishmeal high-plant protein feeds even when all known essential nutrients, including amino acids, are present in the diet above required levels (Gomes et al., 1995; Davies & Morris, 1997; Refstie et al., 2000; Martin et al., 2003; Gomez-Requeni et al., 2004). Although there are several factors blamed for reduced growth of carnivorous fish fed plant protein-based diets, including reduced feed intake, antinutrients in plant products, a lack of anabolic steroids in fishmeal, unidentified nutrient deficiencies and an imbalance of essential amino acids (Gatlin et al., 2007; Glencross et al., 2007; Krogdahl et al., 2010), recent research suggests other factors may be partially responsible for this effect. Plant proteins generally have less lysine, methionine and threonine compared to fishmeal and often deficient for the dietary requirements of fish (NRC, 2011). To correct deficiencies in plant protein-based diets, amino acid supplements are added (NRC, 2011). However, evidence suggests that this approach may cause an imbalance of amino acids in blood plasma associated with delayed digestion and absorption of amino acids of plant origin compared to fishmeal (Boirie et al., 1997; Ambardekar et al., 2009; Larsen et al., 2012). Protein synthesis in cells requires all essential amino acids to be available at the moment proteins are synthesized; if one essential amino acid is not present in sufficient amounts, remaining amino acids are alternatively metabolized for energy (NRC,

2011). This may result in lower protein retention efficiency and increased protein turnover, a common observation when fish are fed plant-based feeds (Davies & Morris, 1997; Refstie et al., 2000; Martin et al., 2003; Ambardekar et al., 2009).

A rainbow trout strain has been developed using selective breeding based on growth performance when fed an all-plant protein feed for 12 years (six generations) at the University of Idaho in collaboration with the US Department of Agriculture's Agricultural Research Service. The selected strain grows rapidly and efficiently when fed all plant-protein feeds containing 45% soy products, unlike unselected trout that exhibit 10-15% lower growth and feed efficiencies than selected trout (Overturf et al., 2013). It is therefore logical that the selected strain could be considered as a model to explore and identify physiological parameters associated with improved plant protein utilization in carnivorous fish.

The major aim of the present study was to identify physiological mechanisms associated with digestion responsible for the improved performance of the selected strain when fed an all-plant protein, soy-based diet. To answer this aim, we performed two series of experiments. In the first experiment we investigated if selection influenced nutrient digestibility which in turn could be responsible for the improved trait, by comparing the selected strain with a non-selected fast-growing strain of rainbow trout when fed a fishmeal-based diet versus the selection diet (all-plant protein soy-based). In the second experiment both rainbow trout strains were fed five practical ingredients (fishmeal and four plant proteins) and a plant protein mixture with or without amino acid supplementation. Temporal plasma amino acid patterns were measured over time to investigate if plasma amino acid temporal patterns at the absorption site (HPV) and from the systemic blood (CV) could be used as a tool to assess alternate ingredients, if the results obtained can be used as predictors when the alternate ingredients are used in a blend and if supplementing a plant protein mixture with certain amino acids can influence the absorption and utilization of the other amino acids. Finally, we tested the hypothesis that the improved protein utilization and growth demonstrated by the selected strain when fed an all-plant protein soy-based diet was the result of a synchronized amino acid uptake.

3.2 Materials and Methods

3.2.1 Experiment 1: Digestibility Trial

3.2.1.1 Experimental Diets

In vivo digestibility values for fishmeal (FMD) and plant meal – based (PMD) diets in two different strains were determined by feeding selected and non-selected groups of rainbow trout the experimental diets containing 0.1% yttrium oxide as an indigestible inert marker (Table 3.1). Diets were prepared at the Hagerman Fish Culture Experiment Station (HFCES) by cold-pelleted using a California pellet mill fitted with a 4 mm die. The pellets were forced-air dried at 37 °C for 48 h to less than 10% moisture. Samples of each diet were collected for analysis.

3.2.1.2 Fish and Feeding

Rainbow trout from brood stock (House Creek and ARS/KO strains) maintained at HFCES were used in the digestibility study, which was run concurrently with a fish feeding trial. Sixteen groups of 35 fish (average body weight 228 g) were stocked into sixteen 450-L tanks supplied with constant temperature spring water (15°C). Each diet was randomly assigned to two replicate tanks of fish per strain. Fish were fed their respective diets twice daily to apparent satiation for 8 days. Photoperiod was maintained at a constant 14 h light: 10 h dark with a timer controlling fluorescent lights. After four days of acclimation to the experimental diets, on day 5 and 9, fish in each tank were lightly anaesthetized using tricaine methanosulfonate (MS-222, 100 mg/L, buffered to pH 7.0), removed from water, and feces gently expelled using light pressure on the abdomen near the vent, a process called stripping. Care was taken to avoid contamination of feces with urine from the fish. Fecal samples were collected in aluminum pans and pooled by tank. Two strippings generated 71-112 g wet fecal samples which was equivalent to 11-14 g dry feces per tank and enough material for subsequent chemical analysis. Feces were frozen between strippings. All protocols used in the digestibility trial were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee.

3.2.1.3 Chemical Analysis

Experimental feeds and fecal samples (for both strains) were analyzed for proximate composition, mineral, amino acids and energy content. Samples were dried in a convection oven at 105°C for 12 h to determine moisture level according to AOAC (2002). Dried samples were finely ground by mortar and pestle and analyzed for crude protein (total nitrogen x 6.25) using combustion method with a nitrogen determinator (TruSpec N, LECO Corporation, St. Joseph, MI). Crude lipid was analyzed by subjecting samples to acid hydrolysis using an ANKOM HCL hydrolysis system (ANKOM Technology, Macedon, NY) and extracting them with petroleum ether using an ANKOM XT15 extractor. Ash was analyzed by incineration at 550°C in a muffle furnace for 5 h. Energy content of samples was determined using an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company Inc., Moline, IL). Macro-minerals were measured in feeds and feces samples from the digestibility trial by inductively-coupled plasma (ICP) analysis at the University of Idaho Analytical Sciences Laboratory (UI-ASL), Moscow, ID. Yttrium was measured in feeds and feces from the digestibility trial. Amino acid levels in feeds and fecal samples were analyzed with an amino acid analyzer (Hitachi Amino Acid Analyzer L-8800) by the University of Missouri's Agricultural Experiment Station Chemical Laboratories, Columbia, MO. Chemical analyses were done in duplicate.

Apparent Digestibility Coefficients (ADCs) of diets in each strain: the ADC values for dry matter, organic matter, protein, amino acids, lipid, energy and minerals, including phosphorus, were calculated using the following formula described by Bureau et al. (2003):

$$\text{ADC diets} = 1 - [(F/D) \times (Di/Fi)]$$

where D = % nutrient of diet, F = % nutrient of feces, Di = % digestion indicator of diet, Fi = % digestion indicator of feces.

3.2.2 Experiment 2

3.2.2.1 Experimental Fish and Dietary Treatments

Two strains of rainbow trout were used, a genetically selected for improved performances on plant protein-based diet (ARS/KO strain) and a non-selected (House Creek strain) (Overturf et al., 2013), both strains were held at the HFCES. Three hundred and fifty individuals (175 / strain) with an average weight 580 ± 209 g were distributed randomly in 70 tanks (5 individuals/strain/tank). The tank size was 144 L and each tank was supplied with constant temperature spring water (15°C) under a controlled photoperiod (14 h light: 10 h dark). The study was conducted over a period of three weeks such that for every day of sampling 12 tanks per test diet were used (six tanks per strain / five sampling points). Prior to experimental use, the fish were hand fed to apparent satiation with a commercial diet (Skretting, USA). All protocols used in the trial were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee.

3.2.2.2 Diets

Five practical, high-protein ingredients were used in the feeding experiment. Anchovy fish meal (FM); corn protein concentrate (CPC); soybean meal (SBM); soy protein concentrate (SPC); wheat gluten meal (WGM). In addition, a plant-protein soy-based mixture was prepared resembling the protein composition of the diet that the ARS/KO strain was selected on for six generations with (Diet Plus) or without (Diet Minus) the supplementation of free crystalline amino acids (lysine, methionine and threonine) in proportions equal to those used in the selection diet (Table 3.2).

3.2.2.3 Force Feeding

The force-feeding procedure followed that of Ambardekar et al. (2009) with minor modifications. After a period of 48 h fasting and prior to the gavage, trout were lightly anesthetized (40 mg/l MS-222, buffered to pH 7.0) and weighed. Every one of the test ingredients was mixed with two parts water to create slurry and delivered to the fish by stomach intubation at 0.5% of live body weight (ratio of dry ingredient or blend to wet body weight). Each anesthetized fish was then forced fed the diet paste

with a 60 ml syringe attached to a piece of Tygon tubing long enough to reach the stomach of the fish. The tubing was inserted in the mouth through the esophagus to reach the stomach of the fish. After feeding, fish were placed in a vigorously aerated fresh water rinse tank for several minutes and then returned to their holding tank.

3.2.2.4 Blood Sampling

For every treatment, blood samples were only taken from individuals that did not show any sign of slurry regurgitation. Blood sampling points were set at 3, 6, 12, 18 and 24 h post force-feeding. Approximately 5 min before blood sampling, each fish was anesthetized in 100 ppm buffered MS – 222 to heavy sedation, i.e., stage 4 when gill operculum movement slowed. The abdomen was opened, and blood was collected (0.2 to 0.3 ml) from the hepatic portal vein (HPV) with a heparinized winged infusion set (butterfly needle; 12-inch tubing, 23 G and 3/4-inch ultra-thin wall needle) connected to a 1 ml syringe. After gently inverting the syringe 3-4 times for proper mixing, the blood was transferred to a 0.6 ml conical Eppendorf tube on ice. Next, blood was collected (1 to 1.5 ml) from the caudal vein (CV) using a 3-ml heparinized syringe with 22 G 1.5-inch needle. After gently inverting the syringe 3-4 times for proper mixing, the blood was transferred to a 2 ml round bottom Eppendorf tube on ice. Blood samples were centrifuged at 2,000 g for 5 min at 4 °C, and the upper plasma layer collected without red blood cells or buffy coat (white blood cells). Plasma proteins were precipitated by adding 13 µl of sulphosalicylic acid into 130 µl of plasma and mixing by gentle vortex for 5 sec. The samples were then incubated at 4 ^{0}C for 20 min and then centrifuged at 16,000g at 4^{0}C for 15 min. Deproteinized plasma (105 µl) was then mixed with 30 µl of 0.3 M NaOH. Finally, 28 µL of internal standard (2.5 mM norleucine) and 117 µl sodium citrate loading buffer (pH 2.2) were added and mixed by vortex for 5 sec and then transferred to a spinX 0.2 µm filter tube and centrifuged for 2 min at 15,000 g at room temperature. The retained filtered sample was then analyzed using a Biochrom 30 amino acid analyser (Biochrom LTD Cambridge, UK) according to the manufacturer's protocol.

3.2.3 Statistical Analysis

3.2.3.1 Experiment 1

Apparent digestibility coefficient values were analyzed for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test). The interaction of strain and diet effects on dry matter, crude protein, lipid, organic matter, energy and amino acid digestibility were analyzed by two-way ANOVA at a 5% level of significance ($\alpha \leq 0.05$). *Post-hoc* tests (Tukey's HSD test) were performed to identify treatments that differed significantly. Statistical analysis was conducted using Statistica (StatSoft, Tulsa OK, USA).

3.2.3.2 Experiment 2

The five practical ingredients we tested for significant interaction effects of strain and time on plasma amino acid levels. Regarding the plant-protein mixtures with and without amino acid supplementation, we tested if there were significant interaction effects of strain, diet and time on plasma amino acids. Plasma amino acid concentration values were analyzed for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test). When the assumptions for normality and homoscedasticity were met, multifactorial analysis of variance (ANOVA) was performed using Statistica (StatSoft, Tulsa OK, USA). In the case when data were violating the assumptions, a permutational multivariate analysis of variance (PERMANOVA) was performed using Primer 7 (Primer-E Ltd, Plymouth, UK). *Post-hoc* tests (Student-Newman-Keuls test) were performed to identify treatments that significantly differed. Plasma amino acid concentrations were expressed as the mean of three replicate measurements.

3.3 Results

3.3.1 Apparent Digestibility Coefficients (ADCs)

Regarding the apparent digestibility coefficients (Table 3.3), no significant interaction effect between diet and strain was detected. In contrast, a statistically significant effect was detected for dry matter and crude protein with higher levels reflected in the groups fed the plant-protein based diet. The same pattern was also observed with all individual amino acids (IAA and DAA) and the sums (sum of IAAs and TAAs). Interestingly, the only significant strain effect was detected in proline ($p<0.05$).

3.3.2 Plasma Amino Acids

3.3.2.1 Fishmeal

In the HPV, there were significant ($P<0.05$) strain and time interaction on Met, Val, Ile and Leu plasma concentrations with both reaching peak levels for the selected strain at 12 h post-prandially (Table 3.4). A time effect was significant ($P<0.01$) on Thr, Phe, His, Lys and Arg; all peaked also at 12 h post-prandially. In the CV, no interaction was found. Significant differences ($P<0.05$) were detected regarding time (all the amino acids) and strain (only His) main effects (Table 3.5). Thr, Met, Val Ile and Leu peaked at 18 h post-prandially while His, Lys and Arg peaked 12 h and Phe at 6 h. However, Val, Ile, Leu, Met, Thr and His recorded significant higher concentrations between 12 and 18 h, while Lys and Arg reached peak levels between 6 and 12 h. Finally, His was significantly higher for the non-selected strain.

3.3.2.2 Soy Protein Concentrate

In the HPV vein, fish fed SPC showed a significant ($P<0.01$) peak at 6 h for Thr, Val, Ile, Leu, Met, Phe Lys and Arg (Table 3.6). At 18 h a second peak was reached ($P<0.01$) for Thr, Val, Ile and Leu. In the CV no interaction was observed (Table 3.7). The time main effect was significant ($P<0.05$) for all the amino acids except His. A

plateau was observed for all amino acids between 3 and 18 h (except Thr which reached a plateau between 6-18 h) and by 24 h concentrations were below initial baseline levels. Arginine showed a significant peak at the 6 h time point.

3.3.2.3 Soybean Meal

In the HPV, significant interactions ($P<0.01$) were detected for all amino acids except Thr and Met (Table 3.8). All plasma amino acid concentrations peaked at 12 h in the selected strain and higher compared to the non-selected strain. In addition, the non-selected strain showed a peak at 18 h for Phe, His and Arg higher compared to selected strain. A time effect was observed regarding Met, showing constant levels between 3 and 12 h and dropping later on, reaching its lowest concentration at 24 h post-prandial ($P<0.01$). In the CV no interaction was observed (Table 3.9). A significant ($P<0.05$) time effect was observed. Phe and Arg concentrations at 6 h were significantly lower compared to concentrations between 12 and 24 h, while the concentrations of Ile and Leu at 6 h were significantly lower compared to all other time points. In contrast, methionine was the only plasma amino acid that showed the highest concentration only at 3 h compared to the whole monitored period.

3.3.2.4 Corn Protein Concentrate

In the HPV, significant ($P<0.05$) interactions observed regarding Thr and Leu (Table 3.10). The plasma Thr and Leu concentrations in the selected strain peaked at 18 h and were significantly higher compared to the non-selected strain. Time had a significant ($P<0.05$) effect on Val, Ile, Leu and Phe. Val and Ile plasma concentrations dropped significantly at 24 h. In contrast, the plasma concentration of Phe at 18 h was significantly higher compared to the 3 h and 6 h time points. A significant main effect ($P<0.05$) was observed in Val and Lys concentrations with lower values in the selected strain compared to the non-selected strain. In the CV, no interactions were observed (Table 3.11). A significant time effect ($P<0.05$) was observed regarding plasma concentrations of Met, Leu and Phe. All the three amino acids showed a significant

increase in their concentrations at 12 h. Strain had a significant effect (P<0.05) on Val, Ile and Lys plasma concentrations being lower in the selected strain.

3.3.2.5 Wheat Gluten Meal

In the HPV, significant interaction effects (P<0.05) were observed regarding Thr, Val, Ile, Leu and Lys (Table 3.12). All the concentrations of the earlier mentioned amino acids showed higher values at 12 h in the plasma of the selected strain compared to the non-selected strain. Time had a significant effect (P<0.05) on the concentration of Met, Phe, His and Arg, showing a drop of their levels at 18 h post-prandially. Moreover, a significant strain effect (P<0.01) was observed for His, with the selected strain having higher concentration levels compared to the non-selected strain. In the CV, significant interaction (P<0.05) was observed with Val (Table 3.13). Val plasma concentration at 12 h post-prandially was higher in the selected strain compared to the non-selected strain. Regarding Thr, Met, Ile and Leu, their plasma concentrations peaked at 12 h post prandially. Regarding His, Lys and Arg, their concentrations dropped significantly at 18 h.

3.3.2.6 Plant Protein Mixtures

In the HPV, a diet by strain by time interaction was significant (P<0.05) among Thr, Leu, Phe, His and Lys plasma concentrations (Table 3.14). At 3 h the selected strain fed the supplemented blend showed significantly higher concentrations for Thr and His compared to the other treatments, while for Phe and Lys showed significantly higher concentrations compared to the non-selected strain fed the non-AA supplanted blend. Also, at 3 h the selected strain fed the supplemented blend showed significantly higher concentration for Leu compared either to the selected and non-selected strains fed the non-supplemented blend. At 6 h the non-selected strain fed the non-supplemented blend showed significantly higher Leu concentration compared to the selected strain fed the supplemented blend and to non-selected strain fed the non-supplemented blend. At 12 h the non-selected strain showed significantly higher Thr concentration compared to the other groups, also the selected strain fed the non-supplemented blend showed higher Leu concentration compared to the non-selected

fed the non-supplemented blend. At 24 h the non-selected strain fed the supplemented blend showed significantly higher Lys concentration compared to the other groups. A strain by time significant interaction ($P<0.05$) was detected regarding plasma concentrations of Val and Met. At 3 h the selected strain showed a significantly higher concentration of Met compared to the non-selected strain. At 18 h the non-selected strain showed significantly higher plasma concentration for Val compared to the selected strain. A diet by time significant effect ($P<0.05$) was detected regarding Val, at 3 h post-prandially the fish group fed the blend supplemented with amino acids showed significantly higher Val concentrations compared to the group fed the non-supplemented blend. Time had a significant effect ($P<0.01$) on Arg and Ile plasma concentrations. At 24 h post-prandially, both amino acids reached the lowest concentrations. Finally, a significant diet ($P<0.01$) was observed regarding Met and Arg plasma concentrations with the fish fed the supplemented blend showing significantly higher concentrations compared to the non-supplemented blend.

In the CV, a diet by strain by time interaction had a significant effect ($P<0.05$) on Thr, Val, Met, Ile, Leu, His and Lys plasma concentrations (Table 3.15). At 3 h the selected strain fed the supplemented blend showed significantly higher concentrations for Thr and His compared to the other treatments, and Lys was higher compared to the selected and non-selected groups fed the non-supplemented blend. At 6 h the selected strain fed the non-supplemented blend showed higher Leu concentration compared to the non-selected fed also non-supplemented blend. At 6 h the selected strain fed the supplemented diet had higher concentration of Lys compared to the selected strain fed the non-supplemented blend. At 18 h the non-selected strain showed significantly higher plasma concentrations of Thr and Lys compared to all the other groups, while Ile plasma concentration was higher compared to the selected strain fed the supplemented blend. At 24 h the non-selected strain fed the supplemented diet showed significantly higher concentrations for Thr and Lys compared to the other groups and for His, Val and Ile compared to the selected strain fed the non-supplemented diet. Also, regarding Leu, the selected strain fed the non-supplemented blend showed significantly lower values compared to the non-selected

strain fed either the supplemented or non-supplemented blends. Time had a significant effect ($P<0.01$) on Arg plasma concentration with the 24 h being significantly lower than the 6 and 3 h. A diet effect also was detected for arginine with the fish group fed the supplemented blend showing significantly higher plasma concentrations compared to the group fed the non-supplemented blend ($P<0.001$).

3.4 Discussion

To our knowledge this is the first study to explore and provide novel insights into the physiological mechanisms that allowed a carnivorous fish species to adapt and thrive when fed an all-plant protein diet. ADC results demonstrate that genetic selection for improved growth and plant protein utilization in rainbow trout does not affect apparent digestibility of nutrients. Our results showed a diet effect with the all-plant protein-based diet showing higher protein digestibility which is in accordance with other studies (Sanz et al., 1994; Gaylord et al., 2008; Callet et al., 2017). Moreover, Callet et al. (2017) compared a rainbow trout strain after three generations of selection on plant-based diet with a control line strain in a 2x2 factorial design (strain by diet), and did not detect any interaction. However, they found a significant increase in apparent digestibility coefficient of protein and decreased values for energy, lipid, moisture and starch when fish were fed an all plant-based diet compared to a fishmeal-based diet. Although apparent digestibility of nutrients remains an important tool for evaluating ingredient quality, it cannot be considered sufficient to assess metabolic utilization of amino acids because does not provide information regarding specific rates of nutrient absorption and metabolism (Karlsson et al., 2006).

Regarding the postprandial plasma amino acids collected in the HPV, concentrations were elevated compared to the systemic blood amino acid concentration levels found in samples from the CV. In a study conducted by Karlsson et al. (2006) using cannulated rainbow trout and force-fed 1% body weight, they found also the same differences in amino acid concentrations between HPV and dorsal aorta samples, and postulated that blood returning to the sinus venosus from the hepatic circulation is diluted by other systemic venous return in direct proportion to the relative

proportion of hepatic blood flow (Karlsson et al., 2006). In the present study, plasma amino acid profiles were strongly affected by the dietary source and reflected the amino acid composition of every corresponding ingredient tested while also maintaining their relative ratios over time. This finding, is in agreement with other studies on rainbow trout (Murai et al., 1987; Schuhmacher et al., 1997; Yamamoto et al., 1998; Larsen et al., 2012; Rolland et al., 2015). We found significant interactions of strain by time in all the tested ingredients in the HPV except for soy protein concentrate but in contrast, in the CV we did not detect any interaction except for valine in wheat gluten meal. However, plasma amino acid measurements from the CV provide less resolution for the protein digestion rate compared to the HPV and this may be related to hepatic and post-hepatic metabolism in contrast to the intestinal uptake (Karlsson et al., 2006).

The fish force-fed fishmeal showed a peak in the HPV for all the plasma amino acids at 12 h postprandial with the selected strain reaching higher levels compared to the non-selected strain, while in the CV the plasma amino acids peaked at 18 h. However, fishmeal, as expected, showed an overall homogeneous pattern for all the amino acids and that agrees with other studies (Larsen et al., 2012). Studies have shown that replacement of fishmeal with proteins of plant origin had an effect on postprandial plasma amino acid regarding the temporal profile and synchronization (Yamamoto et al., 1998; Larsen et al., 2012). That shift, it is assumed to be caused by antinutritional factors, protein solubility differences, and gastric evacuation rate differences which ultimately affect the digestion rate of plant proteins (Boirie et al., 1997; Yamamoto et al., 1998; Bos et al., 2003). In our study, the non-selected strain showed marked differences in plasma amino acid concentrations when force-fed the plant protein ingredients compared to fishmeal. However, the selected strain showed higher peaks in the HPV at 12 h postprandially when force-fed either soybean meal or wheat gluten meal, while the non-selected strain showed a later peak when fed soybean at 18 h and an earlier peak at 6 h when force-fed the wheat gluten meal. In the CV plasma amino acid did not differ significantly for the two strains fed both ingredients. However, it is considered noteworthy to mention that in the case of wheat

gluten meal the non-selected strain peaked in a very similar pattern compared to the selected strain at 12 h postprandially. Wheat gluten meal postprandial amino acid patterns for both strains were homogeneous and were kind similar to the fishmeal patterns except for lysine content which was lower and this observation is in agreement with previous studies (Schuhmacher et al., 1995; Schuhmacher et al., 1997). However, wheat gluten is considered comparable to fishmeal nutritional value when supplemented with amino acids and research has shown that wheat gluten can replace LT-fishmeal in the diets of rainbow trout (Storebakken et al., 2015). An interaction between strain and time was also detected in the HPV of fish fed corn protein concentrate with the selected strain showing a peak at 18 h postprandially for threonine and leucine. Other amino acids even though not significantly different between strains were higher in concentration at 18 h in the selected strain. In contrast, in the CV the non-selected strain showed significantly higher plasma amino acid concentrations compared to the selected strain. Corn protein concentrate showed overall the lowest concentrations for all the plasma amino acids originating either from the hepatic or caudal veins compared to the other plant protein ingredients, even though its protein content is relatively high (75% protein). Finally, soy protein concentrate was the only ingredient for which interactions were not found and further, no strain effect was detected. The only significant effect found was related to time with two major peaks found in the HPV at 6 and 18 h postprandial, while in the CV a plateau for almost all the amino acids was observed between 6 and 18 h. Soy protein concentrate has been considered one of the most promising plant protein sources to replace fishmeal due to its high protein content and lower antinutritional factor levels. Several studies have reported that high inclusion levels showed comparable results to a fishmeal-based diets (Olli and Krogdahl, 1994; Kaushik et al., 1995; Stickney et al., 1996; Mambrini et al., 1999). Regarding our experiment with the balanced versus unbalanced plant protein mixtures fed to the selected and unselected strains over time, we did find significant interactions. In the HPV, balancing the all-plant protein mixture with supplemental amino acids had an effect not only on the concentrations of all the essential amino acids by increasing them, but notably also on plasma amino acids temporal behavior. The selected strain fed the balanced all-plant protein mixture

showed a peak in the amino acid uptake at 3 h postprandially when fed the diet with supplementation compared to the other treatments. However, the supplementation with amino acids, generally, led to an alteration of all dietary essential amino acids uptake in both strains compared to the non-supplemented mixture. Moreover, the selected strain fed the balanced all-plant protein mixture showed a noteworthy difference compared to the unselected strain, specifically, a synchronous and homogenous decreasing pattern for all the essential amino acids over time. Moreover, significant interactions were detected in CV samples for most of the plasma amino acids, with the selected strain maintaining the same synchronized plasma amino acid decreasing pattern as was showed in the HPV. In contrast, the unselected strain showed significantly higher concentrations at 24 h postprandially for arginine, threonine, valine, leucine and very high concentration of lysine compared to the other treatments. The interactions found in the CV demonstrate the strong effect that an all-plant protein mixture can have over the digestive physiology of a carnivorous fish species. Studies using rainbow trout, showed that feeding a plant-protein mixture leads to much less synchronous amino acid uptake compared to when fishmeal is replaced by a single plant protein source, suggesting that different plant-based protein ingredients are diverse in the way they affect the uptake of dietary amino acids (Larsen et al., 2012)**.** Research in swine has demonstrated asynchronous nutrient absorption patterns can be induced by formulating diets using ingredients with different digestion and absorption kinetics (Van den Borne et al., 2007). Furthermore, in the present study, the addition of crystalline amino acids into the all-plant protein mixture affected the plasma concentrations of all amino acids as it did for the uptake reflected in the HPV. Rolland et al. (2016) showed that supplementing a diet with methionine as a single amino acid, it does affect the plasma profiles of other essential amino acids by influencing their concentrations. However, in the present study the selected strain showed a remarkably synchronized dietary amino acid uptake pattern which influenced also the pattern of postprandial appearance of free amino acids in the systemic blood over time. We assume that the fast and homogeneous dietary amino acid uptake in the HPV and the fast-postprandial plasma amino acid disappearance are results of selection for growth on and tolerance of all-plant protein diet. The

selected strain has more rapid growth (~10%) and higher protein retention efficiency (~15%) when fed an all-plant protein diet compared to a non-selected rainbow trout fed a fishmeal-based diet (Overturf et al., 2013). For an optimal amino acid utilization to occur, postprandial plasma amino acid appearance rates do not exceed net protein synthesis capacity. Compared to mammals, in fish the amino acid pool available for protein synthesis derived from intracellular protein degradation is much less (Seiliez et al., 2008); a transient amino acid imbalance would have negative effects on muscle protein turnover. We hypothesize, that plasma amino acid synchronization and increased genetic potential for growth of the selected strain can explain the postprandial plasma amino acid disappearance rate. Our findings are in accordance with a study in growing pigs which was designed to evaluate the effects of synchronized amino acid availability on protein metabolism (Van den borne et al., 2007). These authors found a reduction in protein retention from 57% to 47% in pigs fed a balanced diet characterized by asynchronous temporally amino acid availability.

In conclusion, this is the first study that explored and gave novel insights on the digestive physiology of a carnivorous fish strain genetically selected over six generations for improved plant protein utilization efficiency and growth. Our findings demonstrated that improved performance of the selected strain is associated with a synchronous protein digestion of the plant protein mixture and synchronization of amino acid absorption leading eventually to an improved availability and utilization. Protein digestibility, even though a useful quality assessment tool, does not provide information related to temporal nutrient absorption. In contrast, monitoring temporal plasma amino acid patterns is more useful to assess absorption rate and overall metabolic utilization of amino acids. However, temporal plasma amino acid patterns of single ingredients cannot be used as predictors when the alternate ingredients are used in a blend. Moreover, our results showed that supplementation of amino acids in a diet (at least not balanced) affects the digestion process of the diet in terms of uptake and utilization. Finally, the selected strain ARS / KO proved to be an invaluable and unique model to pursue the discovery of physiological mechanisms necessary for increasing the use of plant proteins in other fish species.

3.5 Bibliography

Ambardekar, A. A., R. C. Reigh, and M. B. Williams. 2009. “Absorption of Amino Acids from Intact Dietary Proteins and Purified Amino Acid Supplements Follows Different Time-Courses in Channel Catfish (*Ictalurus punctatus*).” *Aquaculture* 291(3–4):179–87.

AOAC (Association of Official Analytical Chemists). 2002. Official Methods of Analysis of the Association of Analytical Chemists, 17th Edition. *Association of Official Analytical Chemists*, Washington, DC, USA.

Barrows, F. T., T. G. Gaylord, D. A. J. Stone, and C. E. Smith. 2007. “Effect of Protein Source and Nutrient Density on Growth Efficiency, Histology and Plasma Amino Acid Concentration of Rainbow Trout (*Oncorhynchus mykiss Walbaum*).” *Aquaculture Research* 38(16):1747–58.

Boirie, Y., M. Dangin, P. Gachon, M. P. Vasson, J. L. Maubois, and B. Beaufrere. 1997. “Slow and Fast Dietary Proteins Differently Modulate Postprandial Protein Accretion.” *Proceedings of the National Academy of Sciences* 94(26):14930–35.

Bos, C., C. C. Metges, C. Gaudichon, K. J. Petzke, M. E. Pueyo, C. Morens, J. Everwand, R. Benamouzig, and D. Tomé. 2003. “Postprandial Kinetics of Dietary Amino Acids Are the Main Determinant of Their Metabolism after Soy or Milk Protein Ingestion in Humans.” *The Journal of Nutrition* 133(5):1308–15.

Bureau, D. P., S. J. Kaushik, and C. Y. Cho. 2002. “Bioenergetics.” Pp. 1-59 in: Fish Nutrition, 3rd Edition, J.E. Halver and R.W. Hardy (editors). *Academic Press Inc*., New York, NY.

Bureau, D. P. and P. M. Encarnação. 2006. “Adequately Defining the Amino Acid Requirements of Fish : The Case Example of Lysine.” International De Nutricional Acuicola; *Avances En Nutricional Acuicola* 29–54.

Callet, T., F. Médale, L. Larroquet, A. Surget, P. Aguirre, T. Kerneis, L. Labbé, E. Quillet, I. Geurden, S. Skiba-Cassy, and M. Dupont-Nivet. 2017. “Successful Selection of Rainbow Trout (*Oncorhynchus mykiss*) on Their Ability to Grow with

a Diet Completely Devoid of Fishmeal and Fish Oil, and Correlated Changes in Nutritional Traits." *PLoS ONE* 12(10):1–21.

Davies, S. J. and P. C. Morris. 1997. "Influence of Multiple Amino Acid Supplementation on the Performance of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), Fed Soya Based Diets." *Aquaculture Research* 28(1):65–74.

Gatlin, D. M., F. T. Barrows, P. Brown, K. Dabrowski, T. G. Gaylord, R. W. Hardy, E. Herman, G. Hu, Å. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E. J. Souza, D. Stone, R. Wilson, and E. Wurtele. 2007. "Expanding the Utilization of Sustainable Plant Products in Aquafeeds: A Review." *Aquaculture Research* 38(6):551–79.

Glencross, B. D., M. Booth, and G. L. Allan. 2007. "A Feed Is Only as Good as Its Ingredients - A Review of Ingredient Evaluation Strategies for Aquaculture Feeds." *Aquaculture Nutrition* 13(1):17–34.

Gomes, E. F., P. Rema, and S. J. Kaushik. 1995. "Replacement of Fish Meal by Plant Proteins in the Diet of Rainbow Trout (*Oncorhynchus mykiss*): Digestibility and Growth Performance." *Aquaculture* 130(2–3):177–86.

Gómez-Requeni, P., M. Mingarro, J. a. Calduch-Giner, F. Médale, S. a M. Martin, D. F. Houlihan, S. Kaushik, and J. Pérez-Sánchez. 2004. "Protein Growth Performance, Amino Acid Utilisation and Somatotropic Axis Responsiveness to Fish Meal Replacement by Plant Protein Sources in Gilthead Sea Bream (*Sparus aurata*)." *Aquaculture* 232(1–4):493–510.

Hardy, R. W. 2010. "Utilization of Plant Proteins in Fish Diets: Effects of Global Demand and Supplies of Fishmeal." *Aquaculture Research* 41(5):770–76.

Karlsson, A., E. J. Eliason, L. T. Mydland, A. P. Farrell, and A. Kiessling. 2006. "Postprandial Changes in Plasma Free Amino Acid Levels Obtained Simultaneously from the Hepatic Portal Vein and the Dorsal Aorta in Rainbow Trout (*Oncorhynchus mykiss*)." *Journal of Experimental Biology* 209(24):4885–94.

Kaushik, S. J., J. P. Cravedi, J. P. Lalles, J. Sumpter, B. Fauconneau, and M. Laroche. 1995. “Partial or Total Replacement of Fish Meal by Soybean Protein on Growth, Protein Utilization, Potential Estrogenic or Antigenic Effects, Cholesterolemia and Flesh Quality in Rainbow Trout, *Oncorhynchus mykiss*.” *Aquaculture* 133(3–4):257–74.

Kaushik, S. J. and I. Seiliez. 2010. “Protein and Amino Acid Nutrition and Metabolism in Fish: Current Knowledge and Future Needs.” *Aquaculture Research* 41(3):322–32.

Krogdahl, Å., M. Penn, J. Thorsen, S. Refstie, and A. Bakke. 2010. “Important Antinutrients in Plant Feedstuffs for Aquaculture: An Update on Recent Findings Regarding Responses in Salmonids.” *Aquaculture Research* 41(3):333–44.

Larsen, B. K., J. Dalsgaard, and P. B. Pedersen. 2012. “Effects of Plant Proteins on Postprandial, Free Plasma Amino Acid Concentrations in Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 326–329:90–98.

Mambrini, M., A. J. Roem, J. P. Carvèdi, J. P. Lallès, and S. J. Kaushik. 1999. “Effects of Replacing Fish Meal with Soy Protein Concentrate and of DL-Methionine Supplementation in High-Energy, Extruded Diets on the Growth and Nutrient Utilization of Rainbow Trout, *Oncorhynchus mykiss*.” *Journal of Animal Science* 77(11):2990.

Martin, S. A. M., O. Vilhelmsson, F. Médale, P. Watt, S. Kaushik, and D. F. Houlihan. 2003. “Proteomic Sensitivity to Dietary Manipulations in Rainbow Trout.” *Biochimica et Biophysica Acta* 1651(1–2):17–29.

Murai, T., Ogata, H., Hirasawa, Y., Akiyama, T. & Nose, T. 1987. Portal absorption and hepatic uptake of amino acids in rainbow trout force-fed diets containing casein or crystalline amino acids. *Nippon Suisan Gakkaishi*, 53, 1847–1859.

National Research Council (NRC). 2011. Nutrient Requirements of Fish and Shrimp. *National Academic Press*, Washington, DC.

Naylor, R. L., R. W. Hardy, D. P. Bureau, A. Chiu, M. Elliott, A. P. Farrell, I. Forster, D.M. Gatlin, R. J. Goldburg, K. Hua, and P. D. Nichols. 2009. “Feeding

Aquaculture in an Era of Finite Resources." *Proceedings of the National Academy of Sciences of the United States of America* 106(36):15103–10.

Olli, J.J. and Å. Krogdahi. 1994. "Nutritive Value of Four Soybean Products as Protein Sources in Diets for Rainbow Trout (*Oncorhynchus mykiss* Walbaum) Reared in Fresh Water." *Acta Agriculturae Scandinavica, Section A - Animal Science* 44(3):185–92.

Overturf, K., F. T. Barrows, and R. W. Hardy. 2013. "Effect and Interaction of Rainbow Trout Strain (*Oncorhynchus mykiss*) and Diet Type on Growth and Nutrient Retention." *Aquaculture Research* 44(4):604–11.

Refstie, S., O. J. Korsoen, T. Storebakken, G. Baeverfjord, I. Lein, and a. J. Roem. 2000. "Differing Nutritional Responses to Dietary Soybean Meal in Rainbow Trout (*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*)." Aquaculture 190(1–2):49–63.

Rolland, M., J. P. Feekings, J. Dalsgaard, J. Holm, and P. V. Skov. 2016. "Modelling the Effects of Dietary Methionine Level and Form on Postprandial Plasma Essential Amino Acid Profiles in Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture Nutrition* 22(6):1185–1201.

Rolland, M., B. K. Larsen, J. Holm, J. Dalsgaard, and P. V. Skov. 2015. "Effect of Plant Proteins and Crystalline Amino Acid Supplementation on Postprandial Plasma Amino Acid Profiles and Metabolic Response in Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture International* 23(4):1071–87.

Sanz, A., A. E. Morales, M. de la Higuera, and G. Gardenete. 1994. "Sunflower Meal Compared with Soybean Meals as Partial Substitutes for Fish Meal in Rainbow Trout (*Oncorhynchus mykiss*) Diets: Protein and Energy Utilization." *Aquaculture* 128(3–4):287–300.

Schuhmacher, A., J. Schoen, M. Goldberg, and J. M. Gropp. 1995. "Plasma Amino Acid Levels in Rainbow Trout (*Oncorhynchus mykiss*)." *Journal of Applied Ichthyology* 11(3–4):309–16.

Schuhmacher, A., C. Wax, and J. M. Gropp. 1997. “Plasma Amino Acids in Rainbow Trout (*Oncorhynchus mykiss*) Fed Intact Protein or a Crystalline Amino Acid Diet.” *Aquaculture* 151(1–4):15–28.

Seiliez, I., S. Panserat, S. Skiba-Cassy, A. Fricot, C. Vachot, S. Kaushik, and S. Tesseraud. 2008. “Feeding Status Regulates the Polyubiquitination Step of the Ubiquitin-Proteasome-Dependent Proteolysis in Rainbow Trout (*Oncorhynchus mykiss*) Muscle.” *The Journal of Nutrition* 138(3):487–91.

Stickney, R. R., R.W. Hardy, K. Koch, R. Harrold, D. Seawright, and K. C. Massee. 1996. “The Effects of Substituting Selected Oilseed Protein Concentrates for Fish Meal in Rainbow Trout *Oncorhynchus mykiss* Diets.” *Journal of the World Aquaculture Society* 27(1):57–63.

Storebakken, T., Y. Zhang, J. Ma, M. Øverland, L. T. Mydland, O. F. Kraugerud, E. Apper, and A. Feneuil. 2015. “Feed Technological and Nutritional Properties of Hydrolyzed Wheat Gluten When Used as a Main Source of Protein in Extruded Diets for Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 448:214–18.

Yamamoto, T., T. Unuma, Akiyama, T. 1998. “Postprandial changes in plasma free amino acid concentrations of rainbow trout fed diets containing different protein sources. ” *Fish. Sci*. 64 (3), 474–481

van den Borne, J. J. G. C., Schrama, J. W., Heetkamp, M. J. W., Verstegen, M. W. A., & Gerrits, W. J. J. 2007. “Synchronising the availability of amino acids and glucose increases protein retention in pigs. ” *Animal*, 1(05), 666.

Zhang, Y., M. Øverland, K. D. Shearer, M. Sørensen, L. T. Mydland, and T. Storebakken. 2012. “Optimizing Plant Protein Combinations in Fish Meal-Free Diets for Rainbow Trout (*Oncorhynchus mykiss*) by a Mixture Model.” *Aquaculture* 360–361:25–36.

Table 3.1. Composition of the experimental diets (g/100g)

Ingredient	**PMD**	**FMD**
Soy protein concentrate	25.63	15.00
Soybean meal	19.55	
Corn protein concentrate	17.54	10.00
Wheat gluten meal	4.07	7.00
Wheat starch	8.81	18.00
Fish meal		33.00
Fish oil	15.70	14.00
L-Lysine	1.40	
DL-Methionine	0.38	
Threonine	0.20	
Taurine	0.50	
Dicalcium phosphate	3.33	1.20
Potassium chloride	0.56	
Sodium chloride	0.28	
Magnesium oxide	0.05	
Stay-C	0.20	0.20
Choline chloride	0.60	0.60
Yttrium oxide	0.1	0.10
Trace mineral premix	0.10	0.10
Vitamin premix 702	1.00	0.80

Table 3.2. Composition of the experimental plant-protein mixtures (g/100g)

Ingredient	**Selection Diet**	**Diet Minus**	**Diet Plus**
Soy protein concentrate	25.63	25.63	25.63
Soybean meal	19.55	19.55	19.55
Corn protein concentrate	17.54	17.54	17.54
Wheat gluten meal	4.07	4.07	4.07
Wheat starch	8.91		
Fish oil	15.70		
L-Lysine	1.40		1.40
DL-Methionine	0.38		0.38
Threonine	0.20		0.20
Taurine	0.50		
Mono-dicalcium phosphate	3.33		
Potassium chloride	0.56		
Sodium chloride	0.28		
Magnesium oxide	0.05		
Stay-C	0.20		
Choline chloride	0.60		
Astaxanthin	0.06		
Trace mineral premix	0.10		
Vitamin premix 702	1.00		

Table 3.3. Apparent digestibility coefficients

	Plant Based Diet		Fishmeal Based Diet		DIET x STRAIN	DIET	STRAIN
	SEL	NON SEL	SEL	NON SEL	P-value	P-value	P-value
Dry Matter	76.4±0.2	77.6±0.3	73.2±1.2	74.2±1.6	**ns**	**P<0.05**	**ns**
Crude Protein	93.6±0.1	93.5±0.4	85.3±0.7	86.5±0.7	**ns**	**P<0.001**	**ns**
Lipid	98.0±0.2	97.3±0.1	98.0±0.5	96.1±0.8	**ns**	**ns**	**ns**
Organic Matter	80.2±0.2	81.3±0.4	79.6±1.1	80.5±1.4	**ns**	**ns**	**ns**
Energy	84.1±0.1	84.9±0.3	83.0±1.0	82.9±1.5	**ns**	**ns**	**ns**
Alanine	96.2±0.1	95.6±0.4	90.6±0.5	90.6±0.7	**ns**	**P<0.001**	**ns**
Arginine	98.4±0.1	98.3±0.2	92.8±0.5	93.2±0.6	**ns**	**P<0.001**	**ns**
Aspartic Acid*	93.3±0.3	93.0±0.3	86.0±0.8	87.6±0.7	**ns**	**P<0.001**	**ns**
Cysteine	91.2±0.7	91.8±0.1	81.2±1.0	84.4±1.3	**ns**	**P<0.001**	**ns**
Glutamic Acid*	97.3±0.1	96.6±0.3	92.3±0.4	92.4±0.6	**ns**	**P<0.001**	**ns**
Glycine	92.1±0.2	91.4±0.4	82.2±0.7	82.1±1.0	**ns**	**P<0.001**	**ns**
Histidine	96.5±0.2	96.2±0.2	93.2±0.4	93.6±0.5	**ns**	**P<0.01**	**ns**
Isoleucine	95.7±0.1	95.1±0.3	91.1±0.5	91.3±0.7	**ns**	**P<0.001**	**ns**
Leucine	96.9±0.1	96.3±0.3	93.7±0.4	93.9±0.5	**ns**	**P<0.01**	**ns**
Lysine	97.3±0.2	97.4±0.1	92.4±0.3	93.6±0.5	**ns**	**P<0.001**	**ns**
Methionine	97.4±0.2	97.4±0.1	91.1±0.3	92.2±0.5	**ns**	**P<0.001**	**ns**
Phenylalanine	96.8±0.0	96.7±0.1	93.2±0.4	93.2±0.6	**ns**	**P<0.001**	**ns**
Proline	95.3±0.2	93.0±0.4	87.8±0.2	86.8±0.7	**ns**	**P<0.001**	**P<0.05**
Serine	95.7±0.2	95.0±0.6	89.1±0.6	89.5±0.8	**ns**	**P<0.001**	**ns**
Threonine	92.9±0.3	92.0±0.3	88.1±0.4	88.8±0.8	**ns**	**P<0.01**	**ns**
Tryptophan	97.0±0.0	97.1±0.0	95.3±0.8	95.4±0.3	**ns**	**P<0.05**	**ns**
Tyrosine	96.8±0.1	96.4±0.1	92.2±0.6	92.2±0.6	**ns**	**P<0.001**	**ns**
Valine	94.2±0.1	93.3±0.5	90.4±0.5	90.8±0.7	**ns**	**P<0.01**	**ns**
Sum AA	95.7±0.2	95.2±0.3	89.7±0.5	90.1±0.7	**ns**	**P<0.001**	**ns**
Sum EAA (10)	96.5±0.1	96.1±0.2	92.1±0.4	92.6±0.6	**ns**	**P<0.001**	**ns**

Table 3.4. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein of two strains of rainbow trout during a 24h period after force feeding of fishmeal. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	420±36	576±35	153±13	249±22a	438±38	123±1	194±14	509±61	266±36
SEL	437±54	636±78	148±19	305±46b	523±76	137±16	196±17	554±83	286±47
P-value	**ns**	**ns**	**ns**	**P<0.05**	**ns**	**ns**	**ns**	**ns**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	277±49^{a}	414±46	103±18	180±31	328±59	110±19^{a}	167±23^{a}	438±105^{a}	251±65^{a}
6H	387±37^{ab}	536±36	139±12	253±18	450±32	128±10^{a}	187±10^{a}	560±48^{a}	296±34^{a}
12H	684±101^{c}	933±153	228±29	484±94	827±148	213±32^{b}	292±12^{b}	987±93^{b}	520±49^{b}
18H	485±43^{b}	732±52	187±9	335±37	580±59	130±13^{a}	191±22^{a}	507±83^{a}	266±45^{a}
24H	869±37^{ab}	497±47	112±24	190±24	315±37	96±10^{a}	167±16^{a}	305±46^{a}	127±28^{a}
P-value	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.01**	**P<0.01**	**P<0.001**	**P<0.001**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	251±55	372±50^{a}	82±11^{ab}	146±44^{a}	270±78^{a}	91±29	143±41	309±178	170±107
NON SEL	6H	425±65	576±55^{abc}	151±11^{ab}	257±39^{ab}	470±65^{abc}	132±19	194±13	611±83	338±57
NON SEL	12H	536±117	710±33^{bc}	183±26^{b}	347±31^{ab}	614±45^{bc}	172±25	280±15	852±23	450±14
NON SEL	18H	459±76	631±55^{abc}	185±19^{b}	269±38^{ab}	475±68^{abc}	117±20	182±22	434±95	234±51
NON SEL	24H	411±57	568±61^{abc}	148±38^{ab}	225±35^{ab}	365±53^{abc}	110±18	185±25	386±55	169±43
SEL	3H	294±82	442±71^{ab}	116±28^{ab}	202±43^{ab}	367±87^{abc}	122±26	183±29	524±129	305±82
SEL	6H	350±35	495±43^{ab}	127±20^{ab}	248±14^{ab}	429±21^{abc}	125±13	180±16	509±42	254±27
SEL	12H	833±53	1155±203^{d}	273±15^{c}	621±122^{c}	1040±197^{d}	253±46	305±18	1122±123	590±65
SEL	18H	510±53	833±20^{c}	188±6^{b}	401±34^{b}	686±43^{c}	142±18	201±43	580±142	298±82
SEL	24H	328±45	427±50^{ab}	75±14^{a}	156±19^{a}	265±40^{ab}	83±6	149±17	224±33	85±19
P-value		**ns**	**P<0.01**	**P<0.05**	**P<0.05**	**P<0.05**	**ns**	**ns**	**ns**	**ns**

Table 3.5. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the caudal vein of two strains of rainbow trout during a 24h period after force feeding of fishmeal. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	349±28	516±28	156±13	220±16	358±27	97±5	174±6^{b}	348±31	190±20
SEL	335±25	559±39	143±11	255±22	415±36	106±5	152±6^{a}	398±35	196±18
P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**P<0.05**	**ns**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	244±17^{a}	411±24^{a}	106±5^{a}	180±12^{a}	287±20^{a}	100±3^{b}	156±6^{ab}	311±16^{ab}	169±8^{bc}
6H	346±42^{ab}	528±28^{b}	153±14^{abc}	248±20^{b}	408±31^{b}	118±5^{b}	170±11^{ab}	436±32^{cd}	253±25de
12H	400±45^{b}	624±34^{c}	170±14^{bc}	284±21^{bc}	461±33^{bc}	107±5^{b}	178±6^{b}	508±42^{d}	246±18^{e}
18H	406±15^{b}	710±34^{c}	188±14^{c}	327±23^{c}	539±35^{c}	111±6^{b}	170±8^{b}	387±34^{bc}	194±11^{cd}
24H	305±32^{ab}	430±25^{a}	127±21^{a}	159±10^{a}	256±20^{a}	74±4^{a}	137±12^{a}	222±30^{a}	99±15^{a}
P-value	**P<0.05**	**P<0.001**	**P<0.01**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.05**	**P<0.001**	**P<0.001**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	257±26	426±15	112±10	185±13	300±34	102±7	169±6	309±34	178±16
NON SEL	6H	353±85	526±54	151±26	240±40	393±64	115±9	181±20	423±67	260±53
NON SEL	12H	383±64	580±40	164±24	250±18	404±26	98±6	182±8	440±58	222±28
NON SEL	18H	400±31	641±4	218±11	275±1	461±4	103±8	181±18	324±23	176±22
NON SEL	24H	340±61	420±44	139±32	155±17	249±30	73±7	158±16	222±24	104±16
SEL	3H	235±26	402±41	103±7	176±20	278±29	99±4	148±5	313±21	164±8
SEL	6H	339±42	531±33	154±15	256±20	423±24	120±4	160±9	449±22	247±13
SEL	12H	417±75	668±48	176±20	317±28	518±40	116±6	174±8	576±26	270±17
SEL	18H	411±21	756±34	168±11	362±15	591±28	117±7	162±7	429±38	206±10
SEL	24H	271±16	440±35	115±32	162±14	264±31	76±4	116±3	223±63	94±29
P-value		**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

Table 3.6. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein of two strains of rainbow trout during a 24h period after force feeding of soy protein concentrate. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	336±23	532±30	67±5	262±20	440±32	140±9	178±13	416±34	314±34
SEL	312±16	498±25	65±6	246±18	407±28	131±9	168±9	387±26	290±27
P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	283±12^{a}	470±31^{a}	79±4^{cd}	231±17^{ab}	381±27^{ab}	150±4^{b}	199±13^{b}	397±6^{b}	315±11^{b}
6H	405±10^{b}	616±29^{b}	88±4^{d}	345±20^{c}	557±30^{c}	185±3^{c}	205±7^{b}	585±25^{c}	494±23^{c}
12H	311±17^{a}	507±28^{ab}	64±4^{b}	252±19^{b}	437±33^{b}	127±9^{b}	156±9^{ab}	419±36^{b}	305±33^{b}
18H	391±28^{b}	594±43^{b}	68±5^{bc}	294±27^{bc}	487±43^{bc}	136±12^{b}	187±18^{b}	411±29^{b}	305±24^{b}
24H	256±21^{a}	424±24^{a}	39±2^{a}	178±14^{a}	304±21^{a}	97±6^{a}	126±7^{a}	260±17^{a}	158±16^{a}
P-value	**P<0.001**	**P<0.01**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	282±20	518±51	72±3	254±28	419±48	152±7	200±24	392±12	306±21
NON SEL	6H	409±19	608±69	86±9	337±46	547±69	187±4	215±5	606±47	524±14
NON SEL	12H	334±17	519±46	67±5	265±31	460±52	139±8	162±10	451±50	341±35
NON SEL	18H	441±37	656±21	79±2	324±10	539±18	151±9	215±23	461±20	345±21
NON SEL	24H	236±23	387±26	36±1	154±13	271±21	88±6	113±5	231±19	124±8
SEL	3H	283±16	423±6	86±4	207±2	343±3	147±3	199±18	402±5	323±9
SEL	6H	402±15	624±13	90±3	352±14	568±22	182±6	194±6	564±24	464±34
SEL	12H	275±7	489±33	58±5	234±12	403±22	109±4	147±19	371±40	249±45
SEL	18H	341±14	532±70	57±1	265±53	435±80	122±21	160±19	362±36	266±29
SEL	24H	276±34	460±30	41±4	202±16	338±25	106±6	140±4	289±15	191±9
P-value		**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

Table 3.7. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the caudal vein of two strains of rainbow trout during a 24h period after force feeding of soy protein concentrate. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	287±22	489±28	69±5	231±17	363±26	112±6	174±18	305±23	237±25
SEL	300±17	495±24	74±7	243±15	377±23	115±5	169±9	352±20	257±16
P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	245±28^{ab}	441±28^{ab}	87±10^{b}	203±13^{ab}	318±25^{ab}	127±7^{b}	220±36	323±41^{b}	220±27
6H	340±19^{c}	514±23^{b}	82±6^{b}	255±15^{b}	396±23^{b}	125±6^{b}	169±7	387±9^{b}	336±12
12H	313±14^{bc}	546±34^{b}	79±3^{b}	276±21^{b}	428±32^{b}	110±6^{b}	173±8	381±23^{b}	272±23
18H	345±28^{c}	554±46^{b}	65±7^{b}	272±27^{b}	424±40^{b}	112±10^{ab}	172±18	317±23^{b}	254±17
24H	209±25^{a}	388±29^{a}	40±3^{a}	168±18^{a}	265±25^{a}	89±7^{a}	113±6	212±23^{a}	133±22
P-value	**P<0.01**	**P<0.05**	**P<0.001**	**P<0.01**	**P<0.05**	**P<0.05**	**ns**	**P<0.01**	**P<0.001**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	203±9	442±39	72±14	195±13	302±27	119±9	229±78	251±10	169±14^{ab}
NON SEL	6H	347±12	528±43	82±12	263±28	409±42	134±9	181±8	385±3	348±20^{c}
NON SEL	12H	307±19	514±54	75±4	260±35	410±56	109±2	161±9	378±46	287±32^{c}
NON SEL	18H	381±40	595±65	76±9	284±36	449±53	114±17	193±32	320±47	274±26^{bc}
NON SEL	24H	196±27	367±33	41±3	153±18	245±28	85±8	107±6	193±23	107±19^{a}
SEL	3H	288±46	440±48	101±10	211±26	334±47	136±9	211±19	395±57	270±28^{bc}
SEL	6H	332±40	500±26	82±6	246±16	382±25	116±2	157±4	389±19	324±15^{c}
SEL	12H	318±23	579±43	83±4	291±27	447±39	112±12	185±7	384±25	257±37^{b}
SEL	18H	309±32	512±69	53±5	260±48	400±69	110±13	151±8	314±19	234±19^{b}
SEL	24H	229±60	419±58	38±7	191±36	295±49	95±16	124±5	240±47	173±31^{ab}
P-value		**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**P<0.05**

Table 3.8. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein of two strains of rainbow trout during a 24h period after force feeding of soybean meal. When interaction is present no superscripts are assigned in main factors

STRAIN	Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
NON SEL	283±12	443±14	60±2	219±10	339±15	134±6	151±6	373±19	239±20
SEL	276±12	423±27	56±2	227±20	339±29	143±10	151±7	347±21	270±22
P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**P<0.05**

TIME	Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
3H	289±31	407±26	61±3^{b}	185±14	288±22	112±4	133±6	363±27	194±20
6H	272±13	379±34	63±3^{b}	183±16	275±26	125±7	151±7	335±30	229±19
12H	311±11	515±33	64±2^{b}	284±28	429±40	175±15	172±11	399±37	333±40
18H	276±18	470±27	53±4^{ab}	266±17	400±26	159±10	159±10	389±38	310±29
24H	251±13	395±20	49±3^{a}	197±11	302±17	124±5	141±6	314±16	207±9
P-value	**ns**	**P<0.01**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.05**	**P<0.001**

STRAIN x TIME		Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
NON SEL	3H	288±51	427±34^{ab}	64±6	187±20^{a}	293±29^{ab}	114±8^{a}	127±8^{a}	395±43^{ab}	155±20^{a}
NON SEL	6H	283±10	446±19^{ab}	68±2	210±11^{ab}	422±20^{ab}	125±8^{a}	165±5^{abc}	389±23^{ab}	236±29^{a}
NON SEL	12H	321±4	452±18^{ab}	64±1	226±7^{ab}	349±14^{ab}	142±1^{ab}	151±4^{ab}	325±6^{a}	250±8^{a}
NON SEL	18H	292±12	507±16^{bc}	60±3	282±7^{b}	430±11^{bc}	173±6^{b}	178±6^{bc}	461±23^{b}	360±16^{b}
NON SEL	24H	233±20	383±30^{ab}	47±4	190±13^{a}	299±23^{ab}	118±5^{a}	134±7^{a}	297±27^{a}	193±7^{a}
SEL	3H	291±46	387±43^{ab}	59±2	183±25^{a}	282±39^{a}	110±5^{a}	138±10^{a}	332±26^{a}	232±14^{a}
SEL	6H	261±25	313±32^{a}	59±5	156±21^{a}	229±31^{a}	124±14^{a}	136±6^{a}	281±32^{a}	223±29^{a}
SEL	12H	300±21	577±32^{c}	65±5	342±24^{c}	510±36^{c}	207±11^{c}	194±12^{c}	473±37^{b}	416±32^{b}
SEL	18H	260±35	33±44^{ab}	46±6	249±35^{ab}	369±49^{ab}	145±16^{a}	140±12^{a}	318±41^{a}	261±37^{a}
SEL	24H	268±13	407±30^{ab}	51±4	204±19^{ab}	306±30^{ab}	129±7^{a}	148±8^{ab}	331±17^{a}	221±14^{a}
P-value	**ns**	**P<0.01**	**ns**	**P<0.01**	**P<0.01**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**

Table 3.9. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the caudal vein of two strains of rainbow trout during a 24h period after force feeding of soybean meal. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	192±8	361±12	51±3	158±7	246±12	99±5	122±5	237±16	146±11
SEL	181±22	355±24	46±3	168±14	251±20	103±5	112±6	220±21	160±12
P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	262±36	403±40	64±5^{b}	168±23^{ab}	256±33^{ab}	87±4^{a}	125±11	260±44	128±15^{ab}
6H	170±14	292±34	50±3^{a}	118±15^{a}	181±25^{a}	83±7^{a}	102±8	183±26	111±17^{a}
12H	181±12	369±21	46±4^{a}	174±14^{ab}	268±21^{b}	112±6^{b}	121±8	208±9	172±9^{b}
18H	175±11	386±11	41±4^{a}	196±9^{b}	295±12^{b}	115±4^{b}	118±12	237±21	177±16^{b}
24H	157±37	345±24	44±3^{a}	160±11^{ab}	244±17^{ab}	105±4^{b}	121±8	257±36	173±18^{b}
P-value	**ns**	**ns**	**P<0.01**	**P<0.05**	**P<0.05**	**P<0.001**	**ns**	**ns**	**P<0.05**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	220±53	365±24	62±11	142±24	219±32	82±1	112±3	259±103	112±3
NON SEL	6H	190±20	357±23	56±2	144±9	223±16	81±9	115±12	223±34	118±33
NON SEL	12H	203±1	347±39	54±4	149±17	237±35	106±6	121±18	195±12	157±9
NON SEL	18H	179±7	387±4	43±3	185±1	288±1	119±5	142±10	268±15	177±27
NON SEL	24H	177±11	349±42	43±5	162±19	254±34	102±4	116±5	247±33	154±12
SEL	3H	290±49	429±66	66±6	185±34	280±50	91±5	133±17	261±53	139±24
SEL	6H	151±13	228±33	43±4	92±19	138±31	85±14	88±4	143±23	104±18
SEL	12H	158±15	390±16	38±3	199±7	299±9	118±9	120±2	221±9	187±6
SEL	18H	172±24	386±23	39±8	207±16	302±25	111±7	94±1	207±32	177±24
SEL	24H	137±79	341±34	44±6	157±14	234±17	109±7	126±17	267±74	191±34
P-value		**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

Table 3.10. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein of two strains of rainbow trout during a 24h period after force feeding of corn protein concentrate. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	210±11	364±19^{b}	73±3	156±11	524±38	122±7	135±6	195±29^{b}	97±8
SEL	212±13	305±21^{a}	77±7	140±14	494±60	134±14	145±6	109±11^{a}	116±9
P-value	**ns**	**P<0.05**	**ns**	**ns**	**ns**	**ns**	**ns**	**P<0.05**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	193±16	336±22^{b}	63±5	139±11^{ab}	338±35	101±10^{a}	142±12	182±28	112±11
6H	214±21	340±28^{ab}	66±4	141±17^{ab}	410±34	110±5^{a}	125±7	166±39	110±11
12H	206±9	352±34^{b}	84±3	166±19^{ab}	624±44	147±13^{ab}	143±6	153±51	105±8
18H	236±29	388±31^{b}	90±12	186±22^{b}	723±64	168±27^{b}	155±12	171±44	121±23
24H	205±5	234±29^{a}	73±11	99±11^{a}	477±79	117±12^{ab}	134±9	71±7	72±7
P-value	**ns**	**P<0.05**	**ns**	**P<0.05**	**P<0.001**	**P<0.05**	**ns**	**ns**	**ns**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	203±33^{a}	366±31	65±7	139±20	322±65^{ab}	91±15	136±25	222±48	110±20
NON SEL	6H	234±37^{a}	395±26	72±6	173±19	478±20^{abc}	116±7	132±11	222±66	111±14
NON SEL	12H	210±16^{a}	375±64	82±3	173±36	606±59^{c}	139±11	134±8	221±90	93±12
NON SEL	18H	191±17^{a}	372±49	73±4	164±24	633±37^{c}	134±17	143±12	191±73	94±27
NON SEL	24H	210±4^{a}	282±18	71±18	119±4	608±54^{c}	134±17	124±15	83±3	63±13
SEL	3H	184±4^{a}	307±26	61±7	139±14	354±41^{a}	110±15	149±9	143±6	114±13
SEL	6H	194±22^{a}	284±13	60±5	108±8	341±28^{a}	103±8	117±9	110±19	109±22
SEL	12H	203±12^{a}	329±34	86±6	158±22	642±77^{c}	154±25	151±6	85±5	116±3
SEL	18H	304±10^{b}	412±39	116±17	219±35	857±80^{d}	220±41	173±20	140±37	161±19
SEL	24H	200±7^{a}	187±5	75±20	80±1	346±23^{ab}	101±9	145±1	60±3	81±1
P-value		**P<0.05**	**ns**	**ns**	**ns**	**P<0.01**	**ns**	**ns**	**ns**	**ns**

Table 3.11. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the caudal vein of two strains of rainbow trout during a 24h period after force feeding of corn protein concentrate. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	202±9	362±16^{b}	73±5	149±9^{b}	518±47	115±8	137±6	173±25^{b}	80±6
SEL	182±11	292±14^{a}	78±7	119±7^{a}	453±49	116±8	139±7	80±8^{a}	81±5
P-value	**ns**	**P<0.01**	**ns**	**P<0.05**	**ns**	**ns**	**ns**	**P<0.01**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	162±19	318±20	54±6^{a}	117±7	267±19^{a}	79±3^{a}	128±16	146±24	81±5
6H	197±13	341±20	64±3^{ab}	132±12	371±30^{a}	99±5^{a}	128±8	150±32	90±9
12H	207±16	349±31	85±6^{b}	152±17	576±27^{b}	132±7^{b}	155±8	154±51	86±12
18H	206±16	339±21	82±5^{b}	147±9	612±26^{b}	141±9^{b}	135±5	121±39	82±10
24H	196±13	288±38	91±13^{b}	122±20	601±103^{b}	127±14^{b}	144±8	62±6	64±6
P-value	**ns**	**ns**	**P<0.05**	**ns**	**P<0.001**	**P<0.001**	**ns**	**ns**	**ns**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	181±22	352±27	58±10	122±10	273±40	76±6	126±21	190±22	86±5
NON SEL	6H	208±21	383±14	64±6	156±9	419±43	100±6	122±4	195±52	82±4
NON SEL	12H	228±27	390±49	89±7	175±28	603±21	136±9	159±17	228±85	94±20
NON SEL	18H	190±2	372±31	73±2	160±12	619±21	127±14	143±4	184±60	84±18
NON SEL	24H	204±25	311±47	80±17	130±25	675±124	135±20	132±13	69±10	54±7
SEL	3H	142±30	284±13	49±5	111±9	261±13	82±1	131±29	101±18	75±8
SEL	6H	186±16	299±10	64±2	107±5	323±16	99±10	134±17	105±15	99±17
SEL	12H	186±12	307±24	80±12	130±12	548±50	127±11	150±3	80±23	79±17
SEL	18H	255±1	306±17	92±6	134±12	604±54	154±8	127±6	59±4	79±11
SEL	24H	188±11	264±68	102±22	115±35	527±179	119±21	155±3	55±3	73±6
P-value		**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

Table 3.12. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the hepatic portal vein of two strains of rainbow trout during a 24h period after force feeding of wheat gluten meal. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	284±30	621±50	93±14	323±40	606±63	183±20	207±37^{a}	156±26	197±43
SEL	380±52	682±70	106±15	369±49	680±84	222±30	246±29^{b}	154±23	214±41
P-value	**P<0.01**	**ns**	**ns**	**ns**	**ns**	**ns**	**P<0.01**	**ns**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	368±26	622±32	118±11^{b}	344±32	622±52	230±22^{bc}	260±28^{bc}	259±15	382±38^{c}
6H	421±40	822±66	154±13^{b}	503±50	866±80	294±33^{c}	364±55^{d}	206±18	312±36^{c}
12H	502±140	869±180	131±28^{b}	472±119	896±211	247±46^{bc}	249±48^{cd}	144±45	190±44^{b}
18H	223±31	499±39	50±4^{a}	222±25	442±39	111±11^{a}	122±12^{ab}	84±11	60±6^{a}
24H	192±28	499±57	49±11^{a}	215±28	446±60	138±35^{ab}	129±5^{a}	67±9	56±1^{a}
P-value	**P<0.001**	**ns**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.05**	**P<0.001**	**P<0.001**	**P<0.001**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	333±30^{ab}	597±53^{a}	105±14	308±28^{ab}	572±59^{a}	216±20	201±15	273±19^{b}	355±56
NON SEL	6H	389±40^{ab}	842±118^{a}	155±20	519±88^{bc}	899±137^{ab}	225±36	377±107	222±22^{b}	323±66
NON SEL	12H	281±102^{ab}	586±165^{a}	96±44	295±109^{ab}	584±213^{a}	205±94	191±76	73±4^{a}	129±61
NON SEL	18H	234±76^{ab}	592±1^{a}	54±1	276±31^{ab}	529±11^{a}	135±20	101±20	88±8^{a}	51±2
NON SEL	24H	163±13^{a}	467±42^{a}	42±7	194±17^{a}	412±40^{a}	123±16	122±6	74±15^{a}	55±2
SEL	3H	403±36^{ab}	647±42^{a}	130±17	380±57^{ab}	671±86^{a}	245±43	319±9	245±22^{b}	408±56
SEL	6H	453±74^{b}	802±87^{a}	152±22	486±66^{b}	834±112^{ab}	340±24	351±56	189±28^{b}	301±45
SEL	12H	723±96^{c}	1152±84^{b}	167±11	650±102^{c}	1208±166^{b}	289±15	307±33	216±47^{b}	252±12
SEL	18H	216±34^{ab}	437±18^{a}	48±6	186±12^{a}	385±30^{a}	104±11	135±12	81±18^{a}	66±8
SEL	24H	220±53^{ab}	531±117^{a}	56±23	236±57^{ab}	479±125^{a}	152±75	135±8	61±13^{a}	57±1
P-value		**P<0.05**	**P<0.05**	**ns**	**P<0.05**	**P<0.05**	**ns**	**ns**	**P<0.05**	**ns**

Table 3.13. Free essential amino acid (except tryptophan) mean concentrations n=3 ± SEM in blood plasma (nmol/mL) collected from the caudal vein of two strains of rainbow trout during a 24h period after force feeding of wheat gluten meal. When interaction is present no superscripts are assigned in main factors

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	317±45	637±52	98±13	339±39	606±63	164±18	209±37	129±22	152±28
SEL	324±48	600±62	95±12	332±42	597±72	174±18	208±24	119±22	170±34
P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	362±36^{b}	549±24	109±5^{b}	279±24^{a}	493±36^{a}	183±9^{ab}	251±34^{b}	214±6^{b}	304±28^{c}
6H	328±37^{b}	646±63	116±11^{b}	367±44^{a}	632±71^{a}	194±9^{ab}	295±56^{b}	145±22^{b}	219±25^{b}
12H	619±63^{c}	997±73	168±8^{c}	583±56^{b}	1038±90^{b}	246±30^{b}	302±26^{b}	187±48^{b}	227±15^{b}
18H	202±19^{a}	539±54	67±12^{a}	273±45^{a}	498±67^{a}	120±11^{a}	116±7^{a}	61±9^{a}	59±12^{a}
24H	174±25^{a}	473±53	50±11^{a}	250±41^{a}	473±71^{a}	128±32^{a}	116±4^{a}	47±5^{a}	43±1^{a}
P-value	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.05**	**P<0.05**	**P<0.05**	**P<0.001**	**P<0.001**	**P<0.001**

STRAIN x TIME		**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	3H	310±7	523±28^{ab}	101±8	231±13	423±27	162±4	178±1	209±1	237±5
NON SEL	6H	356±63	751±82^{bc}	134±8	438±54	744±88	183±3	359±104	181±21	240±40
NON SEL	12H	564±134	892±95^{c}	157±8	515±68	925±91	250±66	312±16	176±98	224±24
NON SEL	18H	228±29	638±67^{ab}	79±22	345±69	605±98	140±20	109±15	71±16	68±26
NON SEL	24H	152±8	430±34^{a}	42±7	190±16	378±32	113±13	110±6	51±8	42±1
SEL	3H	396±54	566±36^{ab}	114±4	311±23	540±35	197±5	300±31	218±11	349±12
SEL	6H	301±46	540±47^{ab}	98±14	295±43	519±72	201±14	232±31	109±26	198±34
SEL	12H	674±12	1102±29^{d}	178±10	651±72	1152±118	242±32	291±60	199±64	229±28
SEL	18H	176±15	441±15^{a}	55±5	201±18	391±35	107±6	124±3	52±7	51±4
SEL	24H	189±43	516±104^{ab}	58±22	310±66	567±123	143±70	122±3	44±7	43±2
P-value		**ns**	**P<0.05**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

Table 3.14. Free essential amino acid (except tryptophan) mean concentrations n=3 ±SEM in blood plasma (nmol/mL) collected from the hepatic portal vein of two strains of rainbow trout during a 24h period after force feeding of protein blend with and without AA supplementation (Thr, Met and Lys). When interaction is present no superscripts are assigned in main factors

DIET	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
MINUS	176±11	296±18	30±2 [a]	132±10	276±20	101±5	85±4	138±19	99±11[a]
PLUS	223±16	328±16	98±6 [b]	151±10	323±16	112±6	105±7	241±25	141±16[b]
P-value	**P<0.001**	**ns**	**P<0.001**	**ns**	**P<0.05**	**P<0.05**	**P<0.01**	**P<0.001**	**P<0.01**

STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	202±13	321±15	63±8	144±9	299±17	100±5	90±5	198±21	105±12
SEL	193±15	301±20	61±9	138±11	297±21	112±6	99±7	174±27	133±16
P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	225±24	339±26	64±13	145±17 [b]	252±32	97±11	102±15	241±43	129±28[b]
6H	206±18	353±29	78±14	167±17 [b]	313±28	119±7	97±5	243±36	151±22[b]
12H	218±21	324±18	72±16	155±13 [b]	352±27	122±10	103±11	180±34	147±21[b]
18H	204±20	310±20	59±12	146±10 [b]	336±19	108±6	87±7	159±23	111±12[ab]
24H	137±16	231±26	38±8	94±13 [a]	241±27	84±4	83±6	108±38	56±14[a]
P-value	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.01**	**P<0.001**	**ns**	**P<0.001**	**P<0.01**

DIET x STRAIN	Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
MINUS NON SEL	179±18	315±27	31±4	140±17	289±28	96±8	80±6	150±33	87±18
MINUS SEL	173±13	281±24	30±3	125±12	265±28	105±7	89±5	127±22	109±15
PLUS NON SEL	224±18	327±15	93±7	148±9	308±20	104±7	99±7	242±20	122±15
PLUS SEL	221±28	329±33	105±11	156±20	342±26	121±10	113±14	240±52	165±31
P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

TIME x DIET	Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
MINUS 3H	191±14	298±20 [a]	37±4	118±11	200±22	79±8	83±9	174±36	92±19
PLUS 3H	277±49	401±44 [b]	106±17	186±29	329±56	120±20	130±33	343±71	186±57
MINUS 6H	214±30	390±47 [b]	40±5	185±28	329±48	126±10	94±6	231±62	150±35
PLUS 6H	198±23	315±32 [ab]	117±12	150±18	298±35	113±9	101±7	255±44	153±32
MINUS 12H	190±22	309±29 [ab]	32±3	143±21	337±48	111±12	91±15	121±26	119±27
PLUS 12H	254±31	343±19 [b]	122±8	169±15	370±18	136±14	119±14	254±50	183±26
MINUS 18H	172±14	298±34 [ab]	25±3	143±17	324±32	107±5	78±7	114±23	93±13
PLUS 18H	236±34	322±25 [ab]	93±4	148±12	349±23	110±11	97±11	203±30	130±19
MINUS 24H	108±11	187±18 [a]	16±4	74±7	204±32	82±4	80±8	42±5	42±9
PLUS 24H	166±25	275±43 [ab]	60±2	115±23	279±39	86±8	86±9	175±66	70±26
P-value	**ns**	**P<0.05**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

DIET x	STRAIN x TIME		Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
Minus	NON SEL	3H	188±6^{abc}	271±27	35±4	106±16	179±34^{a}	67±2^{a}	86±18^{a}	135±28^{abc}	69±28
Minus	NON SEL	6H	278±18^{def}	486±27	48±2	244±16	438±1^{e}	131±23^{abc}	104±12^{a}	366±65^{d}	159±93
Minus	NON SEL	12H	142±14^{ab}	266±7	28±4	107±4	247±17^{abc}	93±9^{abc}	61±1^{a}	82±17^{ab}	72±1
Minus	NON SEL	18H	183±20^{abcd}	337±8	29±3	165±6	344±9^{bcde}	113±1^{abc}	78±12^{a}	144±23^{abc}	105±15
Minus	NON SEL	24H	97±21^{a}	223±30	12±7	87±10	264±27^{abcd}	83±11^{abc}	71±9^{a}	34±5^{a}	29±5
Minus	SEL	3H	194±30^{abcd}	324±24	39±7	131±15	222±26^{ab}	97±3^{abc}	80±7^{a}	212±64^{abcd}	115±21
Minus	SEL	6H	172±27^{abcd}	326±44	35±8	146±23	256±33^{abcd}	123±11^{abc}	86±4^{a}	141±35^{abc}	144±33
Minus	SEL	12H	221±17^{abcd}	338±43	34±3	168±26	397±55de	123±17^{abc}	111±17^{a}	147±35^{abc}	150±34
Minus	SEL	18H	155±16^{abc}	240±75	20±2	111±30	294±91^{abcde}	97±7^{abc}	77±4^{a}	69±16^{ab}	75±20
Minus	SEL	24H	115±14^{a}	163±9	18±6	66±6	164±35^{a}	81±4^{ab}	85±11^{a}	47±7^{a}	51±12
PLUS	NON SEL	3H	201±23^{abcd}	358±16	80±4	155±6	263±12^{abcd}	93±9^{abc}	82±7^{a}	269±27^{bcd}	113±23
PLUS	NON SEL	6H	163±10^{abc}	266±7	105±9	120±1	244±4^{abc}	100±6^{abc}	92±8^{a}	203±33^{abcd}	104±8
PLUS	NON SEL	12H	299±16^{ef}	353±1	134±6	174±10	385±19^{cde}	150±17^{c}	131±25^{a}	255±9^{bcd}	197±4
PLUS	NON SEL	18H	274±46^{bcde}	357±5	95±6	164±3	356±34^{bcde}	94±11^{abc}	99±18^{a}	248±14^{bcd}	130±30
PLUS	NON SEL	24H	200±25^{abcd}	320±55	60±2	137±33	303±60^{abcde}	95±10^{abc}	96±13^{a}	249±86^{bcd}	88±42
PLUS	SEL	3H	353±49^{f}	443±89	132±19	217±57	396±100de	146±29^{bc}	178±43^{b}	416±136^{d}	258±92
PLUS	SEL	6H	251±24^{bcd}	389±32	135±27	193±14	378±33^{cde}	132±14^{abc}	114±1^{a}	332±77^{bcd}	226±29
PLUS	SEL	12H	209±39^{abcd}	334±45	109±1	164±35	354±32^{bcde}	123±23^{abc}	106±14^{a}	252±123^{bcd}	169±61
PLUS	SEL	18H	179±10^{abcd}	270±42	91±7	124±22	338±41^{bcde}	133±6^{abc}	95±13^{a}	136±28^{abc}	129±30
PLUS	SEL	24H	115±4^{a}	208±41	59±5	80±17	242±50^{abc}	73±9^{a}	72±1^{a}	63±8^{a}	44±9

P-value	**P<0.001**	**ns**	**ns**	**ns**	**P<0.01**	**P<0.05**	**P<0.01**	**P<0.05**	**ns**

Table 3.15. Free essential amino acid (except tryptophan) mean concentrations n=3 ±SEM in blood plasma (nmol/mL) collected from the caudal vein of two strains of rainbow trout during a 24h period after force feeding of protein blend with and without AA supplementation (Thr, Met and Lys). When interaction is present no superscripts are assigned in main factors

DIET	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
MINUS	164±8	353±18	37±3	144±9	322±19	105±4	104±5	112±8	105±6[a]
PLUS	216±14	354±17	118±4	149±8	337±14	114±3	129±6	233±22	148±10[b]
P-value	**P<0.001**	**ns**	**P<0.001**	**ns**	**ns**	**ns**	**P<0.01**	**P<0.001**	**P<0.001**
STRAIN	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
NON SEL	212±12	389±16	79±9	164±9	352±16	111±4	119±6	210±21	132±9
SEL	171±11	324±16	77±9	131±7	310±16	109±4	114±6	143±19	121±10
P-value	**P<0.001**	**P<0.01**	**ns**	**P<0.05**	**ns**	**ns**	**ns**	**P<0.001**	**ns**
TIME	**Thr**	**Val**	**Met**	**Ile**	**Leu**	**Phe**	**His**	**Lys**	**Arg**
3H	230±20	410±19	84±11	159±9	292±21	100±7	130±13	233±35	145±20 [b]
6H	180±13	365±30	90±15	152±16	308±29	114±4	115±6	183±26	135±12 [b]
12H	170±15	311±14	77±14	132±8	327±20	115±5	115±8	135±16	117±7 [ab]
18H	191±24	342±24	73±14	147±12	365±20	111±5	111±9	146±27	124±14 [ab]
24H	174±20	332±40	61±13	140±19	360±37	106±8	111±11	169±51	106±18 [a]
P-value	**P<0.001**	**P<0.01**	**P<0.01**	**ns**	**ns**	**ns**	**ns**	**P<0.001**	**P<0.01**

DIET	x STRAIN	Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
MINUS	NON SEL	175±10	386±24	40±4	163±14	349±25	105±6	102±8	140±11	109±9
MINUS	SEL	154±11	323±24	34±4	126±10	296±28	105±6	106±7	88±7	100±8
PLUS	NON SEL	252±17	391±23	121±6	166±12	355±22	116±4	137±6	281±28	157±13
PLUS	SEL	187±19	324±22	117±6	136±10	323±17	112±5	122±10	194±30	141±16
P-value		**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

STRAIN	x TIME	Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
NON SEL	3H	225±17	397±25	77±13	158±12	285±27	96±8	128±11	236±45	125±28
NON SEL	6H	193±16	396±51	88±20	169±28	342±43	113±5	122±8	193±20	130±10
NON SEL	12H	177±29	306±15	83±28	123±10	308±22	120±10	113±19	132±22	116±11
NON SEL	18H	237±40	402±23	82±24	178±12	395±23	108±8	108±14	213±35	149±22
NON SEL	24H	221±27	430±35	58±18	186±19	429±17	125±5	126±22	272±93	135±30
SEL	3H	234±35	420±30	89±18	160±15	298±34	105±11	132±24	230±56	161±28
SEL	6H	168±20	335±30	93±26	135±16	274±36	114±6	108±10	174±47	140±24
SEL	12H	166±18	315±21	73±17	137±12	340±31	112±6	116±8	136±23	118±9
SEL	18H	145±14	283±26	64±17	116±12	334±30	115±8	114±11	79±10	98±12
SEL	24H	137±17	253±40	64±21	103±19	304±56	95±10	100±6	87±21	82±18
P-value		**P<0.05**	**P<0.05**	**ns**	**P<0.05**	**ns**	**ns**	**ns**	**P<0.001**	**ns**

DIET X	TIME	Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
MINUS	3H	196±15	379±28	53±3	139±9	248±19	85±3	116±13	140±21	102±18
PLUS	3H	272±32	447±16	120±8	184±9	345±26	120±9	146±25	344±21	195±23
MINUS	6H	157±21	398±55	41±6	164±32	316±56	111±6	99±8	116±20	111±15
PLUS	6H	204±8	333±20	139±8	140±9	300±21	117±5	131±4	238±30	159±15
MINUS	12H	166±16	333±11	36±4	143±6	361±20	114±8	108±12	107±5	114±6
PLUS	12H	175±27	289±21	118±9	120±14	294±30	117±7	121±12	162±26	121±13
MINUS	18H	157±8	345±21	29±4	147±11	369±25	113±8	102±12	105±16	108±5
PLUS	18H	224±46	340±47	117±9	146±24	360±34	110±8	119±13	187±47	139±28
MINUS	24H	136±23	286±67	21±4	118±32	322±76	107±22	89±10	81±20	81±19
PLUS	24H	205±26	368±48	93±7	157±23	390±32	106±7	129±12	239±81	126±27
P-value		**ns**	**P<0.05**	**ns**	**P<0.05**	**ns**	**ns**	**ns**	**P<0.01**	**ns**

DIET x	STRAIN x	TIME	Thr	Val	Met	Ile	Leu	Phe	His	Lys	Arg
MINUS	NON SEL	3H	208±13^{abcde}	360±19^{abc}	57±5^{c}	144±15^{abc}	253±34^{abc}	83±3	134±20^{ab}	171±33^{abc}	96±37
MINUS	NON SEL	6H	188±35^{abcde}	479±74^{c}	46±10^{bc}	208±49^{c}	402±71de	111±11	108±10^{ab}	154±21^{abc}	123±18
MINUS	NON SEL	12H	138±1^{ab}	319±26^{abc}	36±3^{abc}	131±8^{abc}	321±32^{abcde}	111±13	84±4^{a}	106±15^{ab}	100±3
MINUS	NON SEL	18H	154±10^{abcd}	365±29^{abc}	30±5^{ab}	159±15^{abc}	363±36^{abcde}	109±16	82±6^{a}	138±11^{abc}	111±8
MINUS	NON SEL	24H	174±7^{abcde}	387±60^{bc}	27±4^{ab}	164±35^{abc}	422±3de	135±3	92±24^{ab}	114±19^{ab}	112±10
MINUS	SEL	3H	183±27^{abcde}	397±57^{bc}	49±4^{bc}	134±12^{abc}	244±24^{abc}	86±5	86±3^{a}	109±9^{ab}	109±12
MINUS	SEL	6H	125±9^{ab}	317±56^{abc}	37±7^{abc}	120±29^{abc}	229±56^{ab}	111±8	90±12^{ab}	91±20^{ab}	98±24
MINUS	SEL	12H	185±20^{abcde}	342±9^{abc}	36±7^{abc}	151±4^{abc}	388±9^{cde}	116±12	123±13^{ab}	108±2^{ab}	124±4
MINUS	SEL	18H	161±13^{abcde}	325±31^{abc}	28±7^{ab}	135±14^{abc}	374±42^{abcd}	116±10	122±15^{ab}	73±7^{ab}	106±6
MINUS	SEL	24H	98±18^{a}	185±58^{a}	16±4^{a}	71±27^{a}	222±121^{a}	93±29	86±4^{a}	49±2^{a}	49±15
PLUS	NON SEL	3H	251±35^{cde}	453±8^{c}	107±6de	181±2^{bc}	333±5^{abcde}	114±6	118±3^{ab}	333±40^{ef}	168±28
PLUS	NON SEL	6H	198±9^{abcde}	313±20^{abc}	130±9^{ef}	130±8^{abc}	282±25^{abcd}	116±5	135±5^{ab}	219±18^{bcd}	136±11
PLUS	NON SEL	12H	216±43^{bcde}	293±19^{abc}	130±18^{ef}	116±21^{abc}	296±39^{abcde}	129±16	141±23^{ab}	159±36^{abc}	132±15
PLUS	NON SEL	18H	320±30^{f}	439±18^{bc}	134±8^{f}	196±12^{c}	428±18de	107±8	133±18^{ab}	289±15de	187±31
PLUS	NON SEL	24H	267±6e^{f}	473±5^{c}	89±3^{d}	208±4^{c}	437±40^{e}	120±3	159±5^{b}	431±43^{f}	157±64
PLUS	SEL	3H	285±53^{f}	443±28^{bc}	128±9^{ef}	186±17^{c}	353±46^{abcde}	123±16	201±26^{c}	351±30^{f}	214±32
PLUS	SEL	6H	210±13^{bcde}	354±34^{abc}	148±12^{f}	149±16^{abc}	318±34^{abcde}	118±9	126±5^{ab}	257±61^{cde}	182±22
PLUS	SEL	12H	147±28^{abcd}	287±38^{abc}	110±8de	123±22^{abc}	293±49^{abcde}	108±2	108±10^{ab}	164±43^{abc}	113±20
PLUS	SEL	18H	129±23^{ab}	240±23^{ab}	100±6^{d}	96±12^{b}	293±33^{abcde}	113±15	105±17^{ab}	86±20^{ab}	91±25
PLUS	SEL	24H	163±3^{abcde}	299±40^{abc}	96±12^{d}	124±20^{abc}	359±42^{abcde}	96±6	109±2^{ab}	112±27^{ab}	104±20

P-value	**P<0.01**	**P<0.01**	**P<0.05**	**P<0.01**	**P<0.05**	**ns**	**P<0.01**	**P<0.001**	**ns**

CHAPTER 4

STRAIN AND AMINO ACID SUPPLEMENTATION AFFECT AMINO ACID TRANSPORTERS AND OTHER METABOLIC GENE EXPRESSION IN RAINBOW TROUT

4.1 Introduction

Aquaculture production is playing an increasing role in providing fish for human consumption (FAO, 2018). It has been recognized as the fastest growing animal production sector with an annual average growth of almost 6-7% worldwide (NRC, 2011). Fish feeds represent up to 60% of production costs, with dietary protein accounting for nearly half of the cost of most fish feeds (NRC, 2011). The continuing pursuit of knowledge on alternative protein sources to replace fishmeal in farmed fish diets is essential for continued growth of aquaculture production worldwide and to improve the sustainability of aquaculture. Although plant proteins are the most promising alternative protein ingredient for fish feeds, numerous studies have shown suboptimal fish growth performance and reduced protein retention efficiency when fish are fed low fishmeal - high plant protein feeds, even when all known essential nutrients, including amino acids, are present in the diet above required levels (Gomes et al., 1995; Davies and Morris, 1997; Refstie et al., 2000; Martin et al., 2003; Gomez-Requeni et al., 2004).

Studies focusing on the quality and bioavailable nutrient contributions of feed ingredients have been relative few. Evidence suggests that using plant protein blends supplemented with amino acids may cause an imbalance of amino acids in blood plasma that leads to asynchronous digestion and absorption of plant and free amino acids (Boirie et al., 1997; Ambardekar et al., 2009; Larsen et al., 2012). Supplemental crystalline amino acids are rapidly absorbed by trout and other fish whereas absorption of amino acids from plant proteins is slower, resulting in an imbalance of amino acids in the bloodstream and in tissues after a single meal (Ambardekar et al., 2009). Asynchronous amino acid intestinal absorption may also alter protein synthesis

activity in cells which requires all essential amino acids to be available at the moment proteins are being made. If one essential amino acid is not present in sufficient amounts, remaining amino acids are quickly metabolized for energy (NRC, 2011). This results in lower protein retention efficiency and increased protein turnover, a common finding when fish are fed plant (soy)-based feeds (Davies and Morris, 1997; Refstie et al., 2000; Martin et al., 2003; Ambardekar et al., 2009).

A rainbow trout strain has been developed using selective breeding based on growth performance when fed an all-plant protein feed for 12 years (six generations) at the University of Idaho in collaboration with the US Department of Agriculture's Agricultural Research Service. The selected strain grows rapidly and efficiently when fed all plant-protein feeds containing 45% soy products, unlike non-selected trout that exhibited 10-15% lower growth and feed efficiency (Overturf et al., 2013). In the previous study (Chapter 3) we investigated the temporal plasma amino acid patterns in the hepatic portal and caudal veins in the selected strain compared to a non-selected strain when fed a plant-protein mixture and found that the selected strain showed a synchronous amino acid uptake and also disappearance in systemic blood as a result of a nutritional adaptation. Furthermore, transcriptomic studies using these same strains and diets determined that genes related to strain and differential dietary utilization are differentially regulated in multiple metabolic related pathways (Abernathy et al., 2017). The mechanisms of adaptation of nutrient absorption rates by the small intestine have been found to vary between as well as within species (Karasov, 1988). The two main mechanisms potentially responsible are anatomical (intestine) adaptation and changes in specific transport systems (Karasov, 1988). However, results from our lab did not show any differences between the strains in intestine relative length index indicating that anatomical intestinal adaptation is not a significant mechanism that is responsible for increased performance of the selected trout stain (Brezas et al. unpublished data). To further investigate the results from our previous study regarding the synchronization of the plasma amino acids as result of nutritional adaptation, we conducted an additional study to search for differences in the specific transport systems between the selected and non-selected trout strains

that might explain differences in growth performance when fish were fed the all plant-protein test diet. Samples from three tissues (intestine, liver and muscle) were analyzed for gene expression related to digestive process control, amino acid transporter systems, protein degradation and amino acid metabolism.

4.2 Materials and Methods

4.2.1 Experimental Fish and Dietary Treatments

For the present study two strains of rainbow trout were used, a strain selected for improved performances on plant protein-based diet (ARS/KO strain) and a non-selected (House Creek strain) (Overturf et al., 2013). Both strains were from broodstock maintained at the Hagerman Fish Culture Experiment Station, Idaho, USA. One hundred individuals (50 / strain) with an average weight 580 ± 209 g were distributed randomly in 20 indoor tanks (5 individuals/strain/tank). Tank size was 144 L and each tank was supplied with constant temperature spring water (15°C) under a controlled photoperiod (14 h light: 10 h dark). Prior to the study, trout were hand fed to apparent satiation with a commercial diet (Skretting, USA).

4.2.2 Diets

A plant-protein soy-based mixture was prepared in order to resemble the protein composition of the diet that the ARS/KO strain was selected upon for six generations with (Diet Plus) or without (Diet Minus) supplementation of free crystalline amino acids (lysine, methionine and threonine) in proportions equal to those used in the selection diet (Table 4.1).

4.2.3 Force Feeding

The force-feeding procedure followed that of Ambardekar et al. (2009) with minor modifications. After a period of 48 h fasting and prior to the gavage all the individuals were lightly anesthetized (40 mg/l MS-222) and weighed. Each of the test ingredients was mixed with two parts water to create slurry and delivered to the fish

by stomach intubation at a rate of 0.5% of live body weight (ratio of dry diet to wet body weight). Anesthetized fish were forced fed each ingredient or diet slurry using a 60 ml syringe attached to a piece of Tygon tubing of sufficient length to reach the stomach of the fish. The tubing was inserted past esophagus to reach the stomach of the fish. After intubation, each fish was placed in a vigorously aerated fresh water rinse tank for few moments and then returned to its holding tank.

4.2.4 Tissue Sampling

For every treatment, tissue sampling points were set at 3, 6, 12, 18 and 24 h post force-feeding. Fish were killed by cervical dislocation and tissue samples for gene expression were collected from white muscle (dorsal region), liver and intestine (proximal region), flash frozen in liquid nitrogen and stored at -80°C until analysis. The tissues were only collected from individuals that did not show any sign of slurry regurgitation.

4.2.5 RNA Extraction and cDNA Synthesis

RNA isolation from white muscle (dorsal region), liver and intestine (proximal region) tissues of individual fish were collected at the end of the trial and frozen in liquid nitrogen. Extraction of RNA was performed using TRIZol (Invitrogen) according to the manufacturer's protocol. RNA quality was assessed by running the samples on a 1% formaldehyde agarose gel following standard protocols (Sambrook et al.,1989), while the quantity and purity (260 / 280 nm OD ratio ≥ 1.8) of the RNA were determined using a Thermo Scientific Nanodrop 1000 spectrophotometer. Five µg of total RNA was solubilized in RNase-free water and incubated with Dnase I Ambion, Austin, TX, USA) to remove any DNA present in the samples. Total RNA (2 µg) was reverse transcribed using the High Capacity cDNA archive kit with RNase inhibitor (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. At the end of the reaction, the samples were stored at -20 °C.

4.2.6 Gene Expression with real-time Quantitative PCR

Quantitative PCR reactions were performed on an AB 7500 Real Time Quantitative PCR System using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The concentration of cDNA was 20 ng for each 20 µl PCR reaction. Nuclease-free water was used as negative control. PCR reaction cycle conditions were 95 °C for 30 s followed by 60 °C for 3 min over 40 cycles with an initial denaturation step of 95 °C for 2 min. For each gene, assays were run in duplicate on RNA samples isolated from individual fish. Sequences for primer development of the genes of interest were identified using the Basic Local Alignment Search Tool (BLAST) based searches against the rainbow trout expressed sequence transcript (EST) database from The Gene Index Project (COMPBIO) and sequences found in the GenBank (NCBI). Primers were designed and analyzed using the PrimerQuest and OligoAnalyzer tool available at the web page of Integrated DNA Technologies (IDT). Amplification efficiencies of qPCR reactions for each gene were determined using cDNA generated from a pool of all the samples diluted to 1:10 dilution using DNase and RNase free molecular biology grade water. Target gene expression was normalized to a reference gene expression for specific tissues Elongation Factor 1α (ELF1α) for muscle, Ribosomal Protein L11 (RPL11) for liver and Ribosomal Protein S15 (RPS15) for intestine. Melting dissociation curves were performed to confirm single products were amplified. Relative gene expression was calculated using the ($2^{-\Delta CT}$) method (Livak & Schmittgen 2001). The accession numbers and probe and primer sequences of genes evaluated are provided in Table 4.2.

4.2.7 Statistical Analysis

The plant-protein mixtures with and without amino acid supplementation were tested for statistically significant interaction effects ($a < 0.05$) of strain, diet and time on the expression levels of the genes of interest. Expression values were analyzed for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test). When the assumptions for normality and homoscedasticity were met, then multifactorial analysis of variance (ANOVA) was performed using Statistica (StatSoft, Tulsa, OK,

USA). In case the data sets were violating the assumptions a permutational multivariate analysis of variance (PERMANOVA) was performed using Primer 7 (Primer-E Ltd, Plymouth, UK). Post-hoc tests (*Student Newman Keuls* Test) were performed to identify treatments that differed significantly. Data are reported as the means ± standard errors with n = 3 for each treatment.

4.3 Results

4.3.1 Expression of Intestinal Transporters

For amino acid transporters in the intestine (Table 4.3), a significant ($P<0.05$) interaction for diet by strain by time was found. At 24 h the non-selected strain fed the non-supplemented plant protein mixture showing higher *SLC1A1* expression levels compared to non-selected strain fed the non-supplemented plant protein mixture and the selected strain fed the supplemented plant protein mixture. A significant strain by time effect ($P<0.05$) was also found for *SLC15A1* expression at 6 h and 24 h postprandially. The selected strain showed significantly higher levels of expression compared to the non-selected strain, while at 18 h postprandially the non-selected strain showed significant higher levels of *SLC1A5* expression compared to the selected strain. Diet by time was also found to significantly affect ($P<0.01$) for the expression of *SLC7A9*, *SLC1A5* and *SLC36A1*. Supplementation of the all-plant protein mixture increased significantly at 6 h postprandially the expression of SLC7A9 and *SLC1A5* in the supplemented treatment compared to the non-supplementation treatment, and in addition at 12 h increased expression of *SLC1A5* and *SLC36A1*. Time had a significant effect ($P<0.01$) on the expression of *SLC6A19* with its expression at 24 h being significantly higher compared to its expression between three and six h postprandial. A significant difference was found between strains ($P<0.05$) for the expression levels of *SLC7A9* with the non-selected strain showing higher expression levels compared to the selected strain. Finally, a significant diet ($P<0.001$) effect was observed regarding *SLC15A1* expression levels with supplementation increasing its expression levels.

4.3.2 Expression of Metabolic Regulatory Factors in the Intestine

Regarding the results of the examined metabolic genes related in the intestinal tissue, a significant ($P<0.05$) interaction of strain by diet by time was found regarding *CCK-L* expression (Table 4.4). At 6 h the selected strain gavaged with the supplemented plant-protein mixture showed higher expression of *CCK-L* compared to the non-selected strain fed the non-supplemented mixture. While at 18 h the selected strain fed the non-supplemented mixture showed higher expression compared to the selected strain fed the supplemented mixture and the non-selected strain fed the non-supplemented mixture. Finally, at 24 h the non-selected strain fed the supplemented plant-protein mixture showed higher expression levels of *CCK-L* compared to the selected strain fed the supplemented plant-protein mixture and the non-selected strain fed the non-supplemented mixture.

4.3.3 Expression of Metabolic Related Genes in the Liver

Regarding the results of gene expressions measured in the hepatic tissue (Table 4.4), significant interactions ($P<0.05$) of strain by diet by time were found for *KLF15*. *KLF15* expression was determined to be lower at 3 h in the selected strain fed the non-supplemented mixture compared to the other treatments, at 6 h and 12 h the non-selected strain fed the supplemented mixture showed higher expression levels compared to the other treatments. Furthermore, at 18 h the non-selected strain fed the supplemented mixture showed higher expression levels of *KLF15* compared to the selected strain fed the supplemented and non-supplemented mixtures. Diet by time had also a significant effect ($P<0.05$) on the expression of *GOT*. At 24 h, regardless of strain, fish fed the supplementation feed significantly increased expression of *GOT* compared to the non-supplementation treatment. A significant ($P<0.01$) time effect was found for *GPT* with its expression at 24 h being higher compared to its expression levels between 3 h and 12 h, (regardless of diet and strain). Finally, a significant diet effect ($P<0.001$) was found related to GPT expression with supplementation positively influencing its expression.

4.3.4 Expression of Degradation Genes in the Muscle

For gene expression measured in muscle tissue, significant strain by diet by time interactions (P<0.001) were found for *ATG4b* (Table 4.4). At 3 h, the selected strain fed the supplemented plant-protein mixture showed significantly higher expression levels compared to all the other treatments. At 12 h and 18 h, the non-selected strain fed the non-supplemented plant-protein mixture showed significantly higher expression levels compared to the selected strain fed both mixtures, while at 24 h the non-selected strain fed the supplemented plant-protein mixture showed significantly higher expression levels compared to all the other treatments.

4.4 Discussion

Our study is the first to investigate some of the temporal physiological mechanisms of a strain of rainbow trout selected for growth and improved nutrient utilization when fed an all-plant protein diet compared to a non-selected strain.

The only significant interaction found between the three factors (strain, diet and time) was in *SLC1A1* transcript expression levels. Temporal expression patterns showed dramatic differences between strains and amino acid supplementation status with the selected strain fed the supplemented plant protein mixture showing a distinct decreasing expression pattern over time. Expression of *SLC1A1* was even lower than what was found in the non-selected strain under the same treatment. *SLC1A1* excitatory amino acid transporter 3 (EAAT3) belongs to the X^-_{AG} system which is the predominant sodium-dependent transporter for anionic amino acids such as glutamate and aspartate in the intestinal apical membrane (Fan et al., 2004; Ye et al., 2016). To our knowledge this is the first study to monitor the temporal expression of the EAAT3 transcript in fish.

SLC1A15 peptide transporter 1 (PEPT1) expression levels showed an increase over time for the selected strain. In contrast the non-selected strain showed a down regulation of its expression at 6 h and 24 h postprandial. PEPT1 is considered a low-affinity, high-capacity influx transporter which plays an essential physiological role in

protein assimilation and is a major transporter that for the absorption of tri- and di-peptides (Yang et al., 2013). It can transport 400 dipeptides and 8000 tripeptides (Daniel, 2004; Verri et al., 2011). PEPT1 expression is regulated by a number of factors including hormones, feeding state, protein source, antinutritional factors and selective amino acids including phenylalanine, arginine and lysine (Shiraga et al., 1999; Daniel, 2004; Terova et al., 2013; Song et al., 2017; Wang et al., 2017). Taking into consideration that, approximately 80% of digested proteins are absorbed in the form of di- and tri-peptides, as apposed to free amino acids (Yang et al., 2013), and it's expression regulation is driven mainly by substrate availability (Walker et al., 1998), the differences observed in the present study regarding its temporal expression levels validate further the differences found regarding in amino acid uptake synchronization observed between the strains in plasma amino acids from the hepatic portal vein reported in our previous study (Chapter 3).

Regarding the expression of *SLC1A5* (ASCT2), both strains followed the same pattern over time except that at 18 h postprandially, the non-selected strain showed a significantly higher expression compared to the selected strain. *SLC1A5* is also named alanine – serine – cysteine transporter 2 (ASCT2) transports small neutral amino acids including alanine, serine, cysteine, threonine, glutamine, asparagine, methionine, glycine and leucine (Utsunomiya-Tate et al., 1996; Xu et al., 2017). The intestinal isoform ASCT2 together with B^0AT1 are considered to be the major transporters of neutral amino acids in the brush border membrane (Poncet and Taylor, 2012). Moreover, ASCT2 expression levels are known to be positively affected by feeding state and protein levels in the diet (Wu et al., 2015; Song et al., 2017). The expression levels of *SLC7A9* were influenced only by strain with its expression levels being higher in the non-selected strain versus the selected strain. *SLC7A9* ($b^{0,+}$AT) is the light chain of the heteromeric transporter rBAT/ $b^{0,+}$AT (*SLC3A1*/*SLC7A9*), a major transporter for cationic amino acids and cystine in the apical membrane of the intestine (Broer, 2008). Regulators of $b^{0,+}$AT expression are growth factors, hormones and amino acid availability (Hatzoglou et al., 2004). Our findings regarding the interaction between strain and time effects on the specific transporters, ASCT2 and $b^{0,+}$AT, found

in the present study appear to be due to differences in digestion rate kinetics between the strains and related consequently to the temporal differences found in plasma amino acids from the hepatic portal vein, as reported in our previous study (Chapter 3). Free amino acid supplementation was found to affect the temporal expression of ASCT2, $b^{0,+}$AT and *SLC36A1*, a proton-coupled amino acid transporter1 (PAT1) that mainly mediates the transport of glycine, alanine and imino acid (Bröer, 2008). Temporal expression levels in the fish fed the supplemented plant protein mixture for PAT1 were steady in contrast to the non-supplemented mixture, while the ASCT2 transcript showed a slight increase over time compared to the steep increase between 6 and 12 h found in the non-supplemented mixture fed fish group. We believe that the findings regarding PAT1 and ASCT2 are related to alteration of digestion caused by free amino acid supplementation into the diet, resulting in differences in temporal plasma amino acid availability (Rolland et al., 2016). In contrast expression of $b^{0,+}$AT was affected positively by amino acid supplementation, being higher between 3 and 6 h, an expected result mainly because of lysine supplementation. The only transporter whose expression was affected by time was *SLC6A19* which is the system B^0 neutral amino acid transporter AT1 (B^0AT1) responsible for the uptake of a broad range of neutral amino acids across the brush-border membrane of intestinal cells that is known to be affected by feeding state (Orozco et al., 2018). Our results are in agreement with Nitzan et al. (2017) who reported that time after feeding influenced the expression levels of B^0AT1 in the intestine of Mozambique tilapia and the results of Rimoldi et al. (2015) who reported that fishmeal replacement by vegetable proteins did not show any effect on B^0AT1 expression levels in European sea bass.

In the present study the selected strain fed the supplemented plant protein mixture showed a major significant peak in expression of *CCK-L* transcript at 6 h but consequently decreased and remained constant over time, in contrast the non-selected strain fed the supplemented plant-protein mixture after 12 h postprandially showed a significant increase reaching a peak at 24 h post-feeding. Cholecystokinin (CCK) is known to be a hormone that stimulates the secretion of digestive enzymes in vertebrates (Murashita et al., 2015). CCK in rainbow trout is considered a

coordinator of digestive process and satiety by inhibiting gastric emptying and controlling the contraction of the gallbladder (Aldman et al., 1992; Murashita et al., 2008). Oral administration of CCK antagonists in rainbow trout increased feed consumption, supporting the role that CCK is a mediator of satiety (Gelineau and Boujard, 2001). Jonsson et al. (2006) using rainbow trout reported that CCK plasma profile levels increased after feeding and remained high for at least 6 h. Murashita et al. (2015) found that the water-soluble fraction of fishmeal increased CCK expression in juvenile yellowtail, probably through the action of a variety of small peptides and amino acids in the fraction, suggesting that supplementation of a plant protein-based diet with a CCK stimulating factor can improve feed utilization. Our results showed that supplementing the diet with amino acids altered the expression of CCK in both strains but the selected strain fed the supplemented diet showed an earlier peak in expression of CCK while the non-selected strain showed a delayed peak. Moreover, in rainbow trout the feeding-induced CCK response is considered to be slower than in mammals, inferring slower gastric emptying and nutrient absorption rates (Jonsson et al., 2006). We hypothesize that the results of *CCK-L* transcript expression might explain a mechanism of nutritional adaptation in the selected strain in accordance with a differential strain-specific temporal response of amino acid transporters and our findings on plasma amino acids temporal response (Chapter 3).

Regarding expression levels of liver transaminases, we did not find a strain effect but diet and time effects for both *GPT* and *GOT* transcript expression. However, we found that supplementation of the plant protein mixture increased their expression which was further increased over time. Kirchner et al. (2003) fed rainbow trout for 14 days graded levels of protein without finding any significant difference in alanine and aspartate aminotransferase activities. They hypothesized that there is a lack of adaptation of these enzymes to changes in dietary protein content, because the fish capacity to catabolize amino acid in the liver is the same irrespective of the dietary protein level. In a study conducted by Gomez-Requeni et al. (2004), fishmeal was gradually replaced by plant proteins in diets of sea bream for 12 weeks and hepatic activity of aspartate and alanine aminotransferases were not affected. In contrast,

Hansen et al. (2007) found that increasing plant protein inclusion levels in the diet of Atlantic cod led to a decrease of *GPT* and *GOT* hepatic activity. Rolland et al. (2016) reported that *GPT* expression in the liver of rainbow trout was upregulated with increasing levels of supplemented dietary methionine. The studies mentioned earlier did not monitor the temporal activities of these enzymes.

Taking into consideration that metabolism is a dynamic process, measuring adaptive response systems by monitoring the temporal alterations can provide insight and lead to meaningful conclusions. Rolland et al. (2016) hypothesized that *GPT* upregulation was driven by an increased availability of methionine rather than reflecting amino acid utilization or metabolism per se. We also believe that the differences observed in our study are a consequence of higher amino acid availability when amino acids were added to the plant protein mixture resulting to a higher essential amino acid concentration in plasma (Chapter 3). Krüppel-like factor 15 (*KLF15*) is transcription factor known to contribute to the regulation of hepatic gluconeogenesis (Gray et al., 2007). Takashima et al. (2010) using cultured hepatocytes investigated the role of *KLF15* in the regulation of gluconeogenesis and *KLF15* and whether it participates in the anti-diabetes effects of metformin. The results showed that *KLF15* regulates the expression of genes for gluconeogenic and amino acid degrading enzymes. In the present study, *KLF15* expression in the non-selected strain fed the supplemented diet was upregulated between 6 and 18 h postprandially, differing significantly from the other treatments. In contrast, the selected strain fed either the supplemented or the non-supplemented plant-protein mixture showed almost identical patterns which were characterized by low expression of the transcript. Kirchner et al. (2003) hypothesized that rainbow trout persistent hepatic glucose production was due to the high dietary protein content that is required by this species resulting in an amino acid induction of hepatic gluconeogenesis. When the dietary amino acid profile is deficient in one or more essential amino acids, this will limit the utilization of the other amino acids that are present in excess amounts and force their deamination or catabolism (NRC, 2011). Lansard et al. (2010) using rainbow trout hepatocytes demonstrated that increased available amino acids can induce an

increase in expression of gluconeogenic genes with no alteration in protein synthesis. Even though we did not find any strain effect associated with alanine and aspartate transaminases, the interaction effect on *KLF15* expression observed in the present study and our recent findings regarding the plasma amino acid synchronization indicate that the selection for improved plant protein utilization might have an effect on hepatic metabolism in the selected strain. Regarding autophagy-related 4B cysteine peptidase (*ATG4b*) transcript expression in the muscle we found that the selected strain showed low expression levels over time in general (except at 3 h) when fish were fed either the supplemented or non-supplemented plant protein mixtures. On the other hand, the non-selected strain fed either protein mixture showed various peaks in time. Interestingly, when fish were fed the supplemented plant-protein mixture, increased expression levels between 18 and 24 h were measured. In fish the autophagic-lysosome system has been identified as the major proteolytic system responsible for muscle protein degradation (Seiliez et al., 2014). Autophagy is mediated by several autophagy-related proteins among which Atg4 is the only gene whose activity is characterized essential and highly specific, and in rainbow trout *ATG4b* gene is considered to play a key role in muscle atrophy (Seiliez et al., 2010; Maruyama & Noda, 2018). In addition, Seiliez et al. (2012) using rainbow trout myoblasts observed rapid and highly induced *ATG4b* expression when amino acids were removed from the medium, demonstrating that amino acid availability is involved in moderating its expression. Belghit et al. (2014) investigated the effect that various levels of dietary methionine (deficient, adequate and excess) had on muscle proteolytic pathways of rainbow trout and found that methionine deficiency increased *ATG4b* expression. The results of *ATG4b* expression in the present study are considered further evidence that the improved growth and protein retention the selected strain shows, is a result of a controlled protein digestion rate but also there is an overall different physiological homeostatic control which needs further investigation and elucidation.

In conclusion, the results of our study demonstrate that differences in temporal expression levels of amino acid transporters and other metabolic genes associated

with hepatic and muscle metabolic pathways are explaining partially the improvement in growth and feed utilization that the selected trout exhibit when fed an all-plant protein diet. In order to elucidate in more detail, the physiological mechanisms responsible for the nutritional adaptation leading to the improved traits further research is warranted.

4.5 Bibliography

Aldman, G., D. Grove, and S. Holmgren. 1992. “Duodenal Acidification and Intra-Arterial Injection of CCK8 Increase Gallbladder Motility in the Rainbow Trout, *Oncorhynchus mykiss*.” *General and Comparative Endocrinology* 86(1):20–25.

Ambardekar, A. A., R. C. Reigh, and M. B. Williams. 2009. “Absorption of Amino Acids from Intact Dietary Proteins and Purified Amino Acid Supplements Follows Different Time-Courses in Channel Catfish (*Ictalurus punctatus*).” *Aquaculture* 291(3–4):179–87.

Belghit, I., S. Skiba-Cassy, I. Geurden, K. Dias, A. Surget, S. Kaushik, S. Panserat, and I. Seiliez. 2014. “Dietary Methionine Availability Affects the Main Factors Involved in Muscle Protein Turnover in Rainbow Trout (*Oncorhynchus mykiss*).” *British Journal of Nutrition* 112(04):493–503.

Boirie, Y., M. Dangin, P. Gachon, M. P. Vasson, J. L. Maubois, and B. Beaufrere. 1997. “Slow and Fast Dietary Proteins Differently Modulate Postprandial Protein Accretion.” *Proceedings of the National Academy of Sciences* 94(26):14930–35.

Broer, S. 2008. “Amino Acid Transport Across Mammalian Intestinal and Renal Epithelia.” *Physiological Reviews*.

Daniel, H. 2004. “Molecular and Integrative Physiology of Intestinal Peptide Transport.” *Annual Review of Physiology* 66(1):361–84.

Davies, S. J. and P. C. Morris. 1997. “Influence of Multiple Amino Acid Supplementation on the Performance of Rainbow Trout, *Oncorhynchus mykiss*

(Walbaum), Fed Soya Based Diets.” *Aquaculture Research* 28(1):65–74.

Fan, M. Z., J. C. Matthews, N. M. P. Etienne, B. Stoll, D. Lackeyram, and D. G. Burrin. 2004. “Expression of Apical Membrane l -Glutamate Transporters in Neonatal Porcine Epithelial Cells along the Small Intestinal Crypt-Villus Axis.” *American Journal of Physiology-Gastrointestinal and Liver Physiology* 287(2):G385–98.

Food and Agriculture Organization (FAO). 2018. *World Fisheries and Aquaculture Sofia Report*.

Gelineau, A. and T. Boujard. 2001. “Oral Administration of Cholecystokinin Receptor Antagonists Increase Feed Intake in Rainbow Trout.” *Journal of Fish Biology* 58(3):716–24.

Gomes, E. F., P. Rema, and S. J. Kaushik. 1995. “Replacement of Fish Meal by Plant Proteins in the Diet of Rainbow Trout (*Oncorhynchus mykiss*): Digestibility and Growth Performance.” *Aquaculture* 130(2–3):177–86.

Gómez-Requeni, P., M. Mingarro, J. a. Calduch-Giner, F. Médale, S. a M. Martin, D. F. Houlihan, S. Kaushik, and J. Pérez-Sánchez. 2004. “Protein Growth Performance, Amino Acid Utilisation and Somatotropic Axis Responsiveness to Fish Meal Replacement by Plant Protein Sources in Gilthead Sea Bream (*Sparus aurata*).” *Aquaculture* 232(1–4):493–510.

Hansen, A., G. Rosenlund, Ø. Karlsen, W. Koppe, and G. Hemre. 2007. “Total Replacement of Fish Meal with Plant Proteins in Diets for Atlantic Cod (*Gadus morhua L*.) I — Effects on Growth and Protein Retention.” *Aquaculture* 272(1–4):599–611.

Hatzoglou, M., J. Fernandez, I. Yaman, and E. Closs. 2004. “REGULATION OF CATIONIC AMINO ACID TRANSPORT: The Story of the CAT-1 Transporter.” *Annual Review of Nutrition* 24(1):377–99.

Jönsson, E., A. Forsman, I. E. Einarsdottir, B. Egnér, K. Ruohonen, and B. Thrandur

Björnsson. 2006. “Circulating Levels of Cholecystokinin and Gastrin-Releasing Peptide in Rainbow Trout Fed Different Diets.” *General and Comparative Endocrinology* 148(2):187–94.

Kirchner, S., S. Kaushik, and S. Panserat. 2003. “Low Protein Intake Is Associated with Reduced Hepatic Gluconeogenic Enzyme Expression in Rainbow Trout (*Oncorhynchus mykiss*).” *The Journal of Nutrition* 133(8):2561–64.

Lansard, M., S. Panserat, E. Plagnes-Juan, I. Seiliez, and S. Skiba-Cassy. 2010. “Integration of Insulin and Amino Acid Signals That Regulate Hepatic Metabolism-Related Gene Expression in Rainbow Trout: Role of TOR.” *Amino Acids* 39(3):801–10.

Larsen, B. K., J. Dalsgaard, and P. B. Pedersen. 2012. “Effects of Plant Proteins on Postprandial, Free Plasma Amino Acid Concentrations in Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture* 326–329:90–98.

Livak, K. J. and T. D. Schmittgen. 2001. “Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method.” *Methods* 25(4):402–8.

Martin, S. A. M., O. Vilhelmsson, F. Médale, P. Watt, S. Kaushik, and D. F. Houlihan. 2003. “Proteomic Sensitivity to Dietary Manipulations in Rainbow Trout.” *Biochimica et Biophysica Acta* 1651(1–2):17–29.

Maruyama, T. and N. N. Noda. 2018. “Autophagy-Regulating Protease Atg4: Structure, Function, Regulation and Inhibition.” *The Journal of Antibiotics* 71(1):72–78.

Murashita, K., H. Fukasa, N. Takahashi, N. Hosomi, H. Matsunari, H. Furuita, H. Oku, and T. Yamamoto. 2015. “Effect of Feed Ingredients on Digestive Enzyme Secretion in Fish.” *Bull. Fish. R Es. Agen.* 40(40):69–74.

Murashita, K., H. Fukada, I. Rønnestad, T. Kurokawa, and T. Masumoto. 2008.

"Nutrient Control of Release of Pancreatic Enzymes in Yellowtail (*Seriola quinqueradiata*): Involvement of CCK and PY in the Regulatory Loop." *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 150(4):438–43.

Nitzan, T., P. Rozenberg, and A. Cnaani. 2017. "Differential Expression of Amino-Acid Transporters along the Intestine of Mozambique Tilapia (*Oreochromis mossambicus*) and the Effect of Water Salinity and Time after Feeding." *Aquaculture* 472:71–75.

National Research Council (NRC). 2011. Nutrient Requirements of Fish and Shrimp. *National Academic Press*, Washington, DC.

Orozco, Z. A. Gaye, S. Soma, T. Kaneko, and S. Watanabe. 2018. "Spatial MRNA Expression and Response to Fasting and Refeeding of Neutral Amino Acid Transporters Slc6a18 and Slc6a19a in the Intestinal Epithelium of Mozambique Tilapia." *Frontiers in Physiology* 9:212.

Overturf, K., F. T. Barrows, and R. W. Hardy. 2013. "Effect and Interaction of Rainbow Trout Strain (*Oncorhynchus mykiss*) and Diet Type on Growth and Nutrient Retention." *Aquaculture Research* 44(4):604–11.

Poncet, N. and P. M. Taylor. 2013. "The Role of Amino Acid Transporters in Nutrition." *Current Opinion in Clinical Nutrition and Metabolic Care* 16(1):57–65.

Refstie, S., O. J. Korsoen, T. Storebakken, G. Baeverfjord, I. Lein, and a. J. Roem. 2000. "Differing Nutritional Responses to Dietary Soybean Meal in Rainbow Trout (*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*)." *Aquaculture* 190(1–2):49–63.

Rimoldi, S., E. Bossi, S. Harpaz, A. G. Cattaneo, G. Bernardini, M. Saroglia, and Genciana Terova. 2015. "Intestinal B(0)AT1 (SLC6A19) and PEPT1 (SLC15A1) MRNA Levels in European Sea Bass (*Dicentrarchus labrax*) Reared in Fresh

Water and Fed Fish and Plant Protein Sources." *Journal of Nutritional Science* 4:e21.

Rolland, M., J. P. Feekings, J. Dalsgaard, J. Holm, and P. V. Skov. 2016. "Modelling the Effects of Dietary Methionine Level and Form on Postprandial Plasma Essential Amino Acid Profiles in Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture Nutrition* 22(6):1185–1201.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. "Molecular Cloning: A Laboratory Manual." *Molecular Cloning: A Laboratory Manual.* (Ed. 2).

Seiliez, I., K. Dias, and B. M. Cleveland. 2014. "Contribution of the Autophagy-Lysosomal and Ubiquitin-Proteasomal Proteolytic Systems to Total Proteolysis in Rainbow Trout (*Oncorhynchus mykiss*) Myotubes." *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 307(11):R1330–37.

Seiliez, I., J. Gabillard, M. Riflade, B. Sadoul, K. Dias, J. Avérous, S. Tesseraud, S. Skiba, and S. Panserat. 2012. "Amino Acids Downregulate the Expression of Several Autophagy-Related Genes in Rainbow Trout Myoblasts." *Autophagy* 8(3):364–75.

Seiliez, I., J. Gutierrez, C.Salmerón, S. Skiba-Cassy, C. Chauvin, K. Dias, S. Kaushik, S. Tesseraud, and S. Panserat. 2010. "An in Vivo and in Vitro Assessment of Autophagy-Related Gene Expression in Muscle of Rainbow Trout (*Oncorhynchus mykiss*)." *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 157(3):258–66.

Shiraga, T., K. Miyamoto, H. Tanaka, H. Yamamoto, Y. Taketani, K. Morita, I. Tamai, A.Tsuji, and E. Takeda. 1999. "Cellular and Molecular Mechanisms of Dietary Regulation on Rat Intestinal H+/Peptide Transporter PepT1." *Gastroenterology* 116(2):354–62.

Song, F., D. Xu, H. Zhou, W. Xu, K. Mai, and G. He. 2017. “The Differences in Postprandial Free Amino Acid Concentrations and the Gene Expression of PepT1 and Amino Acid Transporters after Fishmeal Partial Replacement by Meat and Bone Meal in Juvenile Turbot (*Scophthalmus maximus L*.).” *Aquaculture Research* 48(7):3766–81.

Takashima, M., W. Ogawa, K. Hayashi, H. Inoue, S. Kinoshita, Y. Okamoto, H. Sakaue, Y. Wataoka, A. Emi, Y. Senga, Y. Matsuki, E. Watanabe, R. Hiramatsu, and M. Kasuga. 2010. “Role of KLF15 in Regulation of Hepatic Gluconeogenesis and Metformin Action.” *Diabetes* 59(7):1608–15.

Terova, G., L. Robaina, M. Izquierdo, A. Cattaneo, S. Molinari, G. Bernardini, and M. Saroglia. 2013. “PepT1 MRNA Expression Levels in Sea Bream (*Sparus aurata*) Fed Different Plant Protein Sources.” *SpringerPlus* 2(1):17.

Utsunomiya-Tate, N., H. Endou, and Y. Kanai. 1996. “Cloning and Functional Characterization of a System ASC-like Na+-Dependent Neutral Amino Acid Transporter.” *The Journal of Biological Chemistry* 271(25):14883–90.

Verri, T., G. Terova, K. Dabrowski, and M. Saroglia. 2011. “Peptide Transport and Animal Growth: The Fish Paradigm.” *Biology Letters* 7(4):597–600.

Walker, D., D. T. Thwaites, N. L. Simmons, H. J. Gilbert, and B. H. Hirst. 1998. “Substrate Upregulation of the Human Small Intestinal Peptide Transporter, HPepT1.” *The Journal of Physiology* 507(3):697–706.

Wang, J., X. Yan, R. Lu, X. Meng, and G. Nie. 2017. “Peptide Transporter 1 (PepT1) in Fish: A Review.” *Aquaculture and Fisheries* 2(5):193–206.

Wu, L., L. He, Z. Cui, G. Liu, K. Yao, F. Wu, J. Li, and T. Li. 2015. “Effects of Reducing Dietary Protein on the Expression of Nutrition Sensing Genes (Amino Acid Transporters) in Weaned Piglets.” *Journal of Zhejiang University. Science. B* 16(6):496–502.

Xu, D., G. He, K. Mai, Q. Wang, M. Li, H. Zhou, W. Xu, and F. Song. 2017. “Effect of Fish Meal Replacement by Plant Protein Blend on Amino Acid Concentration, Transportation and Metabolism in Juvenile Turbot (*Scophthalmus maximus L*.).” *Aquaculture Nutrition* 23(5):1169–78.

Yang, B., Y. Hu, and D. E. Smith. 2013. “Impact of Peptide Transporter 1 on the Intestinal Absorption and Pharmacokinetics of Valacyclovir after Oral Dose Escalation in Wild-Type and PepT1 Knockout Mice.” *Drug Metabolism and Disposition: The Biological Fate of Chemicals* 41(10):1867–74.

Ye, J., C. Gao, X. Li, C. Jin, D. Wang, G. Shu, W. Wang, X. Kong, K. Yao, H. Yan, and X. Wang. 2016. “EAAT3 Promotes Amino Acid Transport and Proliferation of Porcine Intestinal Epithelial Cells.” *Oncotarget* 7(25):38681–92.

Table 4.1. Composition of the experimental plant-protein mixtures (g/100g)

Ingredient	**Selection Diet**		**Diet Minus**	**Diet Plus**
Soy protein concentrate	25.63		25.63	25.63
Soybean meal	19.55		19.55	19.55
Corn protein concentrate	17.54		17.54	17.54
Wheat gluten meal	4.07		4.07	4.07
Wheat starch	8.91			
Fish oil	15.70			
L-Lysine	1.40			1.40
DL-Methionine	0.38			0.38
Threonine	0.20			0.20
Taurine	0.50			
Mono-dicalcium phosphate	3.33			
Potassium chloride	0.56			
Sodium chloride	0.28			
Magnesium oxide	0.05			
Stay-C	0.20			
Choline chloride	0.60			
Astaxanthin	0.06			
Trace mineral premix	0.10			
Vitamin premix 702	1.00			

Table 4.2. Primer sequences used in qRT-PCR assays

Gene	Accession No.	Primer sequence (listed 5'- 3')
ELF1α	AF498320.1	F: GGTCACCACCTACATCAAGAAG
		R: CCCTTGAACCAGCCCATATT
RPS15	BT074197.1	F: ACAGAGGTGTGGACCTGGAC
		R: AGGCCACGGTTAAGTCTCCT
RPL11	BT074162.1	F: GTGGAGCTAAGGCTGAGGTG
		R: CCCAGTGTCGGAGAAGTTGT
KLF15	BX315319	F: AAGAGCAGCCACCTGAAAG
		R: AGCTCATCTGACCTCGAGAA
GOT2	TC197332	F: GGAGAATGCTGGGAGAAACA
		R: AGAGGGCAGGGAGAGTAAA
CCK-L	NM001124345.1	F: GGTCCCAGCCACAAGATAAA
		R: GAGTACTCCTCGTACTCCTCTG
ATG4b	TC190995	F: CTGCGATGTGGACAGATGAT
		R: GCGTTGAGGATACCGATGTAG
GPT	TC174679	F: CCAGAGTAGACAGTGCATTGAG
		R: CAACCCTCCGCATACACATTA
SLC6A19	TC190581	F: GTACAGAGAGACGCTGAACAC
		R: GCCTCGGATGATGCAGATATAG
SLC1A1	BX866040	F: CCATGACAGTGGTAGAGAAAGG
		R: GTACAAGACAACGCGCAAAG
SLC15A1	EU853718	F: GTTTGAAGACCACCAGGAGAA
		R: GACAGTAGACAGGAGACTACCA
SLC1A5	CA356888	F: CCCATTACTACCGCCAAGAG
		R: CGCCCTGAGTGTACCTTAAA
SLC36A1	CA371778	F: GGCTGAGAAGGCACTCAATA
		R: GTCACTGAGGAAGACGAAGTAG
SLC7A9	TC180876	F: CTTCACCAGGAAGGAACTCAA
		R: CTTGTCTATGATCGGTGCTAGG

Table 4.3. Relative mRNA expression quantities of genes related with amino acid transport in the proximal intestine of rainbow trout fed the experimental diets. Values are expressed as mean ± standard error of the mean with n = 3

Diet	SLC15A1	SLC6A19	SLC1A1	SLC7A9	SLC1A5	SLC36A1
Minus	142±8^{a}	2.8±0.1	149±7	83±8	34±3	84±2
Plus	213±9^{b}	3.3±0.2	123±7	106±8	36±2	87±2
P-value	**P<0.001**	**ns**	**P<0.01**	**P<0.05**	**ns**	**ns**

Strain	SLC15A1	SLC6A19	SLC1A1	SLC7A9	SLC1A5	SLC36A1
NON SEL	165±9	3.2±0.1	143±7	106±8^{b}	37±3	84±2
SEL	190±11	2.9±0.2	131±8	84±8^{a}	34±2	86±2
P-value	**P<0.05**	**ns**	**ns**	**P<0.05**	**ns**	**ns**

Time	SLC15A1	SLC6A19	SLC1A1	SLC7A9	SLC1A5	SLC36A1
3H	163±20	3.8±0.3^{c}	137±10	90±13	21±2	90±4
6H	154±22	3.4±0.4^{bc}	142±10	96±18	26±2	89±2
12H	183±9	2.8±0.1^{ab}	144±13	122±17	44±3	78±4
18H	196±13	2.6±0.2^{ab}	130±9	78±7	48±3	83±3
24H	189±15	2.4±0.2^{a}	129±19	85±7	37±2	84±2
P-value	**ns**	**P<0.01**	**ns**	**ns**	**P<0.001**	**ns**

Diet	Strain	SLC15A1	SLC6A19	SLC1A1	SLC7A9	SLC1A5	SLC36A1
Minus	**NON SEL**	130±9	2.9±0.2	147±10	94±10	36±5	83±3
Minus	**SEL**	154±13	2.6±0.2	150±11	75±12	32±4	84±3
Plus	**NON SEL**	202±10	3.4±0.2	137±9	115±12	37±2	85±2
Plus	**SEL**	224±14	3.1±0.4	108±11	96±11	35±2	88±3
P-value		**ns**	**ns**	**ns**	**ns**	**ns**	**ns**

Diet	Time	SLC15A1	SLC6A19	SLC1A1	SLC7A9	SLC1A5	SLC36A1
Minus	**3H**	111±8	3.4±0.3	129±13	72±13^{ab}	18±2^{a}	91±4^{b}
Minus	**6H**	90±10	2.9±0.3	151±13	49±3^{a}	20±1^{a}	89±2^{b}
Minus	**12H**	176±14	2.7±0.1	139±20	124±27^{bc}	53±4^{e}	68±2^{a}
Minus	**18H**	156±8	2.3±0.3	151±10	75±10^{ab}	50±5^{e}	89±4^{b}
Minus	**24H**	174±16	2.6±0.1	184±16	92±15^{ab}	36±3^{cd}	81±3^{ab}
Plus	**3H**	225±16	4.1±0.5	150±13	108±21^{bc}	25±2^{ab}	89±6^{b}
Plus	**6H**	217±24	3.9±0.6	132±14	151±19^{c}	33±1^{bc}	89±4^{b}
Plus	**12H**	189±13	3.0±0.3	152±15	120±21^{bc}	37±1^{cd}	90±4^{b}
Plus	**18H**	227±16	2.9±0.3	112±10	81±11^{ab}	45±4de	77±4^{ab}
Plus	**24H**	209±28	2.2±0.3	73±9	80±4^{ab}	38±2^{cd}	87±2^{b}
P-value		**ns**	**ns**	**P<0.001**	**P<0.01**	**P<0.001**	**P<0.01**

Strain	Time	SLC15A1	SLC6A19	SLC1A1	SLC7A9	SLC1A5	SLC36A1
NON SEL	**3H**	181±31^{abcd}	4.0±0.3	143±15	92±17	19±3^{a}	93±4
NON SEL	**6H**	119±20^{a}	3.1±0.2	160±11	118±42	26±2^{a}	86±3
NON SEL	**12H**	189±13^{bcd}	2.9±0.2	144±19	136±11	44±4^{c}	74±4
NON SEL	**18H**	185±16^{bcd}	3.0±0.3	137±8	92±10	56±3^{d}	79±5
NON SEL	**24H**	152±9^{abc}	2.7±0.2	122±18	98±11	38±3^{c}	86±3
SEL	**3H**	141±21^{ab}	3.5±0.6	131±13	88±20	23±2^{a}	87±7
SEL	**6H**	189±35^{cd}	3.7±0.6	121±12	83±17	25±4^{ab}	91±4
SEL	**12H**	175±14^{abcd}	2.7±0.1	145±19	110±30	43±4^{c}	83±7
SEL	**18H**	208±22^{cd}	2.2±0.2	123±15	66±7	41±4^{c}	88±4
SEL	**24H**	216±22^{d}	2.1±0.3	135±35	73±6	36±1^{bc}	81±3
P-value		**P<0.05**	**ns**	**ns**	**ns**	**P<0.05**	**ns**

Diet	Strain	Time	SLC15A1	SLC6A19	SLC1A1	SLC7A9	SLC1A5	SLC36A1
Minus	**NON SEL**	**3H**	112±10	3.4±0.6	146±24^{bcd}	80±26	15±3	91±7
Minus	**NON SEL**	**6H**	84±17	2.7±0.2	172±19de	45±1	22±2	92±3
Minus	**NON SEL**	**12H**	168±7	2.7±0.1	121±26^{bcd}	114±2	54±3	67±3
Minus	**NON SEL**	**18H**	146±7	2.8±0.4	142±16^{bcd}	97±5	63±2	85±7
Minus	**NON SEL**	**24H**	150±14	2.7±0.3	157±18^{cde}	126±7	36±5	82±4
Minus	**SEL**	**3H**	110±15	3.2±0.2	112±6^{abcd}	63±9	22±3	91±6
Minus	**SEL**	**6H**	98±7	3.0±0.5	129±9^{bcd}	51±5	18±1	85±2
Minus	**SEL**	**12H**	183±30	2.6±0.2	156±32^{cde}	132±51	52±8	70±3
Minus	**SEL**	**18H**	165±12	2.0±0.3	159±12^{cde}	64±11	41±5	94±3
Minus	**SEL**	**24H**	197±25	2.5±0.1	211±16^{e}	59±3	36±1	80±7
Plus	**NON SEL**	**3H**	249±11	4.5±0.1	138±20^{bcd}	104±25	27±5	95±5
Plus	**NON SEL**	**6H**	166±18	3.4±0.3	149±11^{bcd}	190±2	31±2	79±1
Plus	**NON SEL**	**12H**	205±19	3.2±0.5	174±16de	158±10	36±2	84±4
Plus	**NON SEL**	**18H**	214±14	3.2±0.4	133±5^{bcd}	88±18	49±1	72±5
Plus	**NON SEL**	**24H**	155±1	2.7±0.2	87±6^{ab}	77±6	40±3	90±2
Plus	**SEL**	**3H**	189±1	3.7±1.1	169±4de	112±38	23±2	84±11
Plus	**SEL**	**6H**	256±25	4.4±1.1	110±28a^{bcd}	125±20	35±1	96±5
Plus	**SEL**	**12H**	168±7	2.8±0.2	129±18^{bcd}	81±24	37±2	96±7
Plus	**SEL**	**18H**	240±29	2.5±0.2	96±10^{abc}	69±3	42±7	82±5
Plus	**SEL**	**24H**	235±36	1.8±0.5	59±12^{a}	83±5	37±2	82±2
P-value			**ns**	**ns**	**P<0.05**	**ns**	**ns**	**ns**

Table 4.4. Relative mRNA expression quantities of genes related with digestion process control in the proximal intestine, amino acid metabolism in the liver and protein degradation in the muscle of rainbow trout fed the experimental diets. Values are expressed as mean ± standard error of the mean with n = 3

Strain	**CCK-L**	**GPT**	**GOT**	**KLF15**	**ATG4B**
NON SEL	0.39±0.04	211±13	90±5	5.9±0.5	139±13
SEL	0.39±0.03	200±12	89±6	2.9±0.3	97±21
P-value	**ns**	**ns**	**ns**	**P<0.001**	**P<0.01**

Time	**CCK-L**	**GPT**	**GOT**	**KLF15**	**ATG4B**
3H	0.27±0.05	194±20[a]	67±6	3.5±0.6	194±40
6H	0.39±0.04	167±18[a]	66±7	5.7±0.5	85±10
12H	0.33±0.03	187±16[a]	90±5	6.1±1.2	86±14
18H	0.38±0.05	219±15[ab]	96±6	3.9±0.5	119±28
24H	0.58±0.08	256±18[b]	132±8	2.7±0.4	94±20
P-value	**P<0.001**	**P<0.01**	**P<0.001**	**P<0.001**	**P<0.001**

Diet	**CCK-L**	**GPT**	**GOT**	**KLF15**	**ATG4B**
Minus	0.34±0.03	172±11[a]	89±5	3.7±0.3	106±14
Plus	0.44±0.05	236±10[b]	90±6	5.1±0.6	127±20
P-value	**P<0.05**	**P<0.001**	**ns**	**P<0.001**	**P<0.001**

Diet	**Strain**	**CCK-L**	**GPT**	**GOT**	**KLF15**	**CCK-L**	**ATG4B**
Minus	**NON SEL**	0.28±0.02	187±18	95±7	4.6±0.3	0.28±0.02	146±21
Minus	**SEL**	0.40±0.05	157±12	82±8	2.8±0.5	0.40±0.05	71±13
Plus	**NON SEL**	0.51±0.08	234±16	85±8	7.1±0.9	0.51±0.08	133±16
Plus	**SEL**	0.37±0.04	238±14	95±8	3.0±0.4	0.37±0.04	121±38
P-value		**P<0.01**	**ns**	**ns**	**P<0.01**	**P<0.01**	**P<0.001**

Strain	Time	CCK-L	GPT	GOT	KLF15	ATG4B
NON SEL	**3H**	0.32±0.09	203±23	69±9	5.0±0.4	132±18
NON SEL	**6H**	0.34±0.05	155±17	55±5	6.4±1.1	111±12
NON SEL	**12H**	0.33±0.04	170±19	99±5	8.8±1.5	101±22
NON SEL	**18H**	0.33±0.06	248±23	105±8	5.2±0.5	214±26
NON SEL	**24H**	0.63±0.15	259±31	128±11	3.8±0.5	129±43
SEL	**3H**	0.22±0.03	186±33	66±9	1.8±0.7	280±80
SEL	**6H**	0.43±0.05	179±33	78±13	5.1±0.5	63±7
SEL	**12H**	0.32±0.04	199±23	81±9	2.9±0.5	68±17
SEL	**18H**	0.43±0.08	194±16	86±7	2.4±0.2	38±7
SEL	**24H**	0.53±0.07	252±8	135±12	1.6±0.3	70±11
P-value		**ns**	**ns**	**ns**	**P<0.01**	**P<0.001**

Diet	Time	CCK-L	GPT	GOT	KLF15	ATG4B
Minus	**3H**	0.22±0.02	136±9	63±9^{a}	3.7±1.1	162±15
Minus	**6H**	0.30±0.03	131±14	58±7^{a}	4.9±0.5	73±7
Minus	**12H**	0.33±0.03	139±4	93±7^{abc}	3.7±0.5	101±34
Minus	**18H**	0.39±0.07	202±18	107±8^{bc}	3.3±0.6	120±45
Minus	**24H**	0.48±0.08	249±28	118±11^{c}	2.6±0.4	56±6
Plus	**3H**	0.34±0.11	245±25	71±9^{ab}	3.4±0.7	225±79
Plus	**6H**	0.50±0.04	204±28	73±12^{ab}	6.8±0.9	95±17
Plus	**12H**	0.32±0.04	235±12	87±8^{abc}	8.1±1.8	78±13
Plus	**18H**	0.36±0.07	234±23	85±6^{abc}	4.3±0.8	118±38
Plus	**24H**	0.67±0.14	262±23	148±6^{d}	2.8±0.7	131±31
P-value		**ns**	**ns**	**P<0.05**	**P<0.01**	**P<0.001**

Diet	Strain	Time	CCK-L	GPT	GOT	KLF15	ATG4B
Minus	**NON SEL**	**3H**	0.22±0.03^{a}	162±1	79±12	5.2±0.5^{cd}	174±26de
Minus	**NON SEL**	**6H**	0.24±0.02^{a}	136±26	56±9	4.6±0.3^{cd}	85±4^{abcd}
Minus	**NON SEL**	**12H**	0.37±0.04^{abc}	139±4	106±6	4.8±0.2^{cd}	156±28^{cde}
Minus	**NON SEL**	**18H**	0.24±0.03^{a}	243±3	117±13	4.3±0.9^{bcd}	213±40^{e}
Minus	**NON SEL**	**24H**	0.35±0.04^{abc}	253±53	112±14	3.6±0.2^{bcd}	55±1^{abc}
Minus	**SEL**	**3H**	0.22±0.03^{a}	116±5	48±7	0.5±0.0^{a}	151±16^{bcd}
Minus	**SEL**	**6H**	0.34±0.02^{abc}	123±2	60±12	5.1±0.8^{cd}	64±8^{abc}
Minus	**SEL**	**12H**	0.28±0.05^{abc}	139±7	75±5	2.7±0.5^{abcd}	46±5^{a}
Minus	**SEL**	**18H**	0.58±0.03^{cd}	171±21	97±9	2.4±0.5^{abc}	28±2^{a}
Minus	**SEL**	**24H**	0.58±0.13^{bcd}	244±8	123±18	1.9±0.3^{abc}	57±10^{abc}
Plus	**NON SEL**	**3H**	0.46±0.21^{abc}	235±32	58±12	4.5±0.6^{bcd}	101±8^{abcd}
Plus	**NON SEL**	**6H**	0.45±0.04^{abc}	181±10	54±7	9.0±0.4^{e}	128±10^{abcde}
Plus	**NON SEL**	**12H**	0.30±0.06^{abc}	216±2	90±2	11.9±0.7^{f}	74±17^{abc}
Plus	**NON SEL**	**18H**	0.46±0.11^{abc}	251±43	94±9	5.8±0.5^{d}	214±42^{e}
Plus	**NON SEL**	**24H**	0.83±0.21^{d}	265±41	145±10	3.9±0.9^{bcd}	202±21^{e}
Plus	**SEL**	**3H**	0.22±0.05^{a}	255±43	85±10	2.7±0.9^{abcd}	473±33^{f}
Plus	**SEL**	**6H**	0.56±0.06^{bcd}	221±49	91±18	5.3±0.0^{cd}	62±13^{abc}
Plus	**SEL**	**12H**	0.35±0.06^{abc}	245±17	85±15	3.2±0.9^{abcd}	83±25^{abcd}
Plus	**SEL**	**18H**	0.27±0.06^{ab}	217±22	76±7	2.3±0.1^{abc}	46±10^{ab}
Plus	**SEL**	**24H**	0.46±0.03^{abc}	259±14	150±10	1.3±0.6^{ab}	84±17^{abcd}
P-value			**P<0.05**	**ns**	**ns**	**P<0.001**	**P<0.001**

CHAPTER 5

SYNTHESIS

5.1 Introduction

Aquaculture production now supplies more than half of the fish consumed in the world and is the fastest growing animal production sector with an annual average growth of almost 6-7% worldwide (NRC, 2011). Growth of aquaculture production has been propelled by many factors with the main factor being intensification of production systems. Intensification is defined in the aquaculture context as increasing production from aquaculture systems by water quality management and by provision of high-quality feeds as high levels. Feeds for intensive aquaculture have historically contained high levels of fishmeal and fish oil, expensive marine-derived ingredients produced from finite supplies of wild fish (Naylor et al., 2009). However, fish feed production has increased greatly over the past two decades and global production of fishmeal and oil have remained static. This has greatly increased their prices and forced fish feed producers to reduce fishmeal and fish oil levels in feeds and increase the use of alternative ingredients. Feed costs are a major economic factor determining profitability of aquaculture production, contributing to more than 60% of operating costs, with the protein component of feed constituting the highest portion (NRC, 2011). For economic reasons, replacement of fishmeal is a major objective for the development of sustainable aquaculture.

Plant proteins and especially soybean meal (SBM) are the leading candidate protein ingredients to partially or totally replace fish meal. The major advantages of the plant proteins are favorable protein content and amino acid profile, their chemical consistency and the fact that are economically attractive and sustainably produced (Hardy 1996; Lim et al., 2004). However, plant proteins also present limitations regarding their use for carnivorous species which are related to problems connected with protein digestion, amino acid utilization and metabolic disturbances associated with antinutritional factors (Gatlin et al., 2007; Krogdahl et al., 2010; Blaufuss and

Trushenski, 2011). Replacement is associated with reduced fish performance and protein retention when fish are fed high plant protein feeds even when all known essential nutrients, including amino acids, are present in the diet above required levels (Refstie et al., 2000; Martin et al., 2003; Gomez-Requeni et al., 2004). Evidence suggests that using plant protein blends supplemented or not with amino acids may cause an imbalance of amino acids in blood plasma that leads to asynchronous digestion and absorption of plant and free amino acids (Boirie et al., 1997; Ambardekar et al., 2009; Larsen et al., 2012). Asynchronous amino acid intestinal absorption may also alter protein synthesis activity in cells which requires all essential amino acids to be available at the moment proteins are being made. If one essential amino acid is not present in sufficient amounts, remaining amino acids are quickly metabolized for energy (NRC, 2011). This is hypothesized as the cause of the lower protein retention efficiency and increased protein turnover, a common finding when fish are fed plant (soy)-based feeds (Martin et al., 2003; Ambardekar et al., 2009; Larsen et al., 2012).

A novel approach to investigate mechanisms associated with inferior performance of rainbow trout fed plant-based feeds high in soy proteins is now possible by utilizing a fish strain that exhibits desirable performance traits when fed an all plant protein, high-soy diet as a positive control model (Abernathy et al., 2017). After 12 years and over $15 million of research investment, rainbow trout families have been developed at the University of Idaho that are such a model. These fish were selected for performance using an all plant-protein diet for six generations. This diet is essentially considered as the “diet of the future” having all the characteristics which are required (sustainability and economic viability) to be used in intensive production of carnivorous marine fish and salmonids. Thus, the selected strain is a unique model to identify physiological parameters associated with plant-protein (soy) utilization in fish. They grow rapidly when fed all plant-protein feeds containing 45% soy products without exhibiting distal enteritis, unlike unselected trout that also exhibited 10-15% lower growth and feed efficiencies (Overturf et al., 2013). By exploring the molecular/metabolic characteristics exhibited by the selected line of rainbow trout and

comparing them to those of non-selected trout, novel insights to make significant advancements can be achieved.

5.2 Digestibility and Plasma Amino Acids

A digestibility study was conducted to investigate whether selection had any influence on nutrient digestibility, which in its turn could be partially responsible for the improved traits exhibited by the selected trout strain. The results did not show an interactive effect between genotype and diet, or a genotype main effect on the apparent digestibility coefficients. In contrast, results indicated a main diet effect with the all-plant protein-based diet showing higher protein digestibility, validating further results from previous studies (Gaylord et al., 2008; Callet et al., 2017). Findings clearly validate using apparent digestibility of nutrients as an evaluation tool for ingredient quality. However, findings also show that measuring apparent digestibility is not sufficient to assess protein digestion rates and metabolic utilization of amino acids because it does not provide information regarding specific rates of nutrient absorption and metabolism (Karlsson et al., 2006). Furthermore, results demonstrated that other physiological mechanisms are responsible for the differences in fish performance between selected and non-selected trout strains fed an all plant-protein, high-soy diet.

A subsequent study was conducted to determine if plasma free amino acid temporal patterns from blood collected at the absorption site (hepatic portal vein) and from systemic blood (caudal vein) could be used as a tool to evaluate the effects of alternate ingredients on fish digestive physiology that explain differences in trout strain performance. For the above-mentioned purpose two rainbow trout strains (selected and nonelected) were force-fed (by gavage) five practical ingredients (fishmeal, soybean meal, soy protein concentrate, corn protein concentrate and wheat gluten meal) and the temporal plasma amino acid patterns were measured over time at the absorption site and from the systemic blood. A comparison of the results of the plasma free amino acid temporal patterns, obtained from the hepatic portal vein and the caudal vein demonstrated that plasma amino acid measurements from the hepatic portal vein are more robust and provide higher resolution. These findings are in

accordance with results of Karlsson et al. (2006) who found also the same magnitude of differences in amino acid concentrations between hepatic portal vein and dorsal aorta samples using cannulated rainbow trout force-fed 1% body weight. They postulated that the differences observed were due to hepatic and post-hepatic metabolism in contrast to intestinal uptake. Our results showed that overall plasma amino acid profiles were strongly affected by dietary ingredients and reflected the amino acid composition of every corresponding ingredient tested while also maintaining their relative ratios over time. This finding, is in agreement with other studies on rainbow trout (Murai et al., 1987; Schuhmacher et al., 1997; Yamamoto et al., 1998; Larsen et al., 2012; Rolland et al., 2015). Fishmeal showed an overall homogeneous pattern for all amino acids without significant differences between the strains. In contrast marked differences in plasma amino acid concentrations compared to fishmeal were found between and within strains when trout were force-fed the plant protein ingredients. Significant interaction effects were found regarding strain by time for all the tested ingredients in the hepatic portal vein except for soy protein concentrate, indicating differences related to digestive rates between strains. In contrast, in the caudal vein no interaction detected for any plant ingredient except for valine in wheat gluten meal. The fact that differences in plasma amino acid patterns in either trout strain fed soy protein concentrate did not show any significant differences may explain findings from several studies which reported that high inclusion levels of wheat gluten meal in feeds showed comparable results to a fishmeal-based diets (Olli and Krogdahl, 1994; Kaushik et al., 1995; Stickney et al., 1996; Mambrini et al., 1999). Although wheat gluten meal postprandial amino acid patterns for both strains differed in concentration, they were found overall homogeneous and resembled fishmeal patterns except for lysine content which was lower, in agreement with previous studies (Schuhmacher et al., 1995; Schuhmacher et al., 1997). Regarding soybean meal the selected strain showed a peak at 12 h while the non-selected strain showed a more delayed peak at 18 hours, while in the case of corn protein concentrate the selected strain was the only one to show defined peaks at 18 h postprandially. The results obtained from the use of the five practical ingredients demonstrated that plasma amino acids from the hepatic portal vein are a

valuable tool to evaluate the effects of candidate alternate proteins on fish digestive physiology. Furthermore, our findings showed that each ingredient can affect digestive physiology of the fish in a different manner due to differences in antinutritional factor content, protein solubility, and gastric evacuation rates (Boirie et al., 1997; Yamamoto et al., 1998; Bos et al., 2003).

Furthermore, we investigated if the results obtained with individual ingredients were additive and could predict fish performance when combined in a blend to replace fishmeal in feeds. Such blends require amino acid supplementation to meet dietary requirements of rainbow trout, so the effects of amino acid supplementation to plant protein blends on absorption and utilization of the other amino acids was also investigated. Finally, we investigated the hypothesis that the improved protein utilization and growth demonstrated by the selected strain when fed an all-plant protein soy-based diet was the result of a synchronized amino acid uptake. To answer these questions two rainbow trout strains (selected and non-selected) were force-fed (by gavage) a plant protein mixture (containing the four plant ingredients) with or without amino acid supplementation (lysine, methionine and threonine) and the temporal plasma amino acid patterns were measured over time at the absorption site and from the systemic blood.

Regarding the supplemented versus the unsupplemented plant protein mixtures fed to the selected and non-selected strains over time, significant strain by diet by time interactions was detected. In more detail, balancing the all-plant protein mixture with supplemental amino acids had an effect not only on the concentrations of all the essential amino acids by increasing them, but notably also on plasma amino acids temporal behavior. To our knowledge this is the first study that monitored the temporal effects of single plant protein ingredients and their mixture on fish digestive physiology using plasma free amino acid concentrations as a response variable. Our results showed that plant protein ingredients with known effects on fish digestive behavior when fed individually do not have any predictable additive effect when fed together as a mixture. Moreover, we found that the addition of crystalline amino acids into the all-plant protein mixture affected the plasma concentrations of all the amino

acids as it did for uptake reflected in the hepatic portal vein. Our findings are in accordance with a study conducted by Rolland et al. (2015) who observed that supplementing a diet with methionine as a single amino acid affected the plasma profiles of other essential amino acids by influencing their concentrations. Finally, a major finding of this study was that the selected strain fed the amino acid supplemented all-plant protein mixture showed a noteworthy difference compared to the unselected strain, specifically, a synchronous and homogenous decreasing pattern for all the essential amino acids over time in the hepatic portal vein. Moreover, significant interactions were detected also in caudal vein for most of the plasma amino acids, with the selected strain maintaining the same synchronized plasma amino acid decreasing pattern as was showed in the hepatic portal vein. Studies using rainbow trout, showed that feeding a plant-protein mixture leads to much less synchronous amino acid uptake compared to when fishmeal is replaced by a single plant protein source suggesting that different plant-based protein ingredients are diverse in the way they affect the uptake of dietary amino acids (Larsen et al., 2012). A study using growing pigs showed that protein retention is influenced by temporal amino acid availability leading eventually to a decrease from 57% to 47% in pigs fed a balanced diet characterized by asynchronous temporally amino acid availability (Van den borne et al., 2007). Moreover, compared to mammals, in fish the amino acid pool available for protein synthesis deriving from intracellular protein degradation is much less (Seiliez et al., 2008); a transient amino acid imbalance would have negative effects on muscle protein turnover. We assume that the homogeneous dietary amino acid uptake in the hepatic portal vein and the fast-postprandial plasma amino acid disappearance are results of nutritional adaptation driven by selection for growth on and tolerance of all-plant protein diet.

5.2.1 Limitations of the Current Studies

The major limitation of the current study is related with the sample size of the second experiment. Great pains were taken in the study to control potential confounding effects associated with differences among fish in feed intake. The use of gavage to deliver a precise dose of each feed ingredient to each fish based upon 0.5%

of body weight eliminated differences in feed intake as a confounding variable. The experimental plan called for analyzing five samples per treatment group at each sampling point, so six fish were gavaged by group to provide an extra fish if needed. However, some fish were excluded from sampling because it was noted at sampling that gavaged feed ingredient material had not left the stomach. Samples were taken from remaining fish as planned but not all samples were suitable for analysis, thereby reducing the number of actual samples analyzed per treatment group per time point to three. This was not anticipated and, as a result, statistical power was lower than planned, making the statistical analysis less likely to detect significant interactive and main effects.

5.3 Gene Expression Analyses

To further investigate the results from the first study regarding synchronization of plasma amino acids as result of nutritional adaptation, a second study was conducted to search for differences in the specific transport systems between the selected and non-selected trout strains that might explain differences in growth performance when fish were fed the all plant-protein test diet. Samples from various tissues (intestine, liver and muscle) were analyzed for specific gene expression analysis related to digestive process control, amino acid transporter systems, protein degradation and amino acid metabolism.

The results showed that expression levels of amino acid transporters were affected by strain, diet and time. Amino acid transporter expression is influenced by many factors with the major one being substrate availability (Walker et al., 1998; Daniel, 2004; Hatzoglou et al., 2004; Wu et al., 2015; Orozco et al., 2018). The anionic amino acid transporter (*SLC1A1)* transcript expression was the only one influenced by interaction of all the factors which in the selected strain fed the supplemented plant protein mixture showed a distinct decreasing expression pattern over time which was lower compared to the other treatments. Furthermore, strain was found to have an effect on the expression levels of the cationic amino acid transporter (*SLC7A9)* and an interactive effect with time on the expression levels of the peptide transporter

(*SLC15A1)* and of the alanine – serine – cysteine transporter 2 (*SLC1A5*). The differences observed between the strains validate the differences found regarding amino acid uptake synchronization due to differences in digestion rate kinetics observed between the strains in plasma amino acids from the hepatic portal vein due reported in the previous study. This is the first study in fish to demonstrate that selection for plant protein utilization in fish can have an effect on the expression levels of four major amino acid transporters. Furthermore, diet was found to have an effect on the expression levels of *SLC15A1* and an interactive effect with time on the expression levels of proton-coupled amino acid transporter 1 (*SLC36A1*), *SLC7A9* and *SLC1A5*. These findings are supporting further the findings from the previous study showing that crystalline amino acid supplementation of a plant protein mixture alters digestion, resulting in differences in temporal plasma amino acid availability (Rolland et al., 2016).

Results showed that supplementing the protein ingredient blend with amino acids altered the expression of cholecystokinin transcript (*CCK-L*) in both strains but the selected strain fed the supplemented diet showed an earlier peak in expression of *CCK* while the non-selected strain showed a delayed peak. CCK in rainbow trout is considered a coordinator of digestive process and satiety by inhibiting gastric emptying and controlling the contraction of the gallbladder (Aldman et al., 1992; Murashita et al., 2008). The results of *CCK* transcript expression might explain a mechanism of nutritional adaptation in the selected strain in accordance with the differential strain-specific temporal response of amino acid transporters found in this study and findings on plasma amino acids temporal response in the previous study.

Regarding expression levels of liver transaminases (*GPT* and *GOT*), we found that supplementation of the plant protein mixture with crystalline amino acids increased their expression which was further increased over time. These findings are in agreement with Rolland et al. (2016) who reported that *GPT* expression in the liver of rainbow trout was upregulated with increasing levels of supplemented dietary methionine, hypothesizing that *GPT* upregulation was driven by an increased availability of methionine rather than reflecting amino acid utilization or metabolism

per se. The differences observed in this study appear to be a consequence of higher amino acid availability when amino acids were added to the plant protein mixture resulting to a higher essential amino acid concentration in plasma.

An interaction effect of strain by diet by time was found regarding Krüppel-like factor 15 (*KLF15*) expression levels with the non-selected strain fed the supplemented diet showing upregulation between 6 and 18 h postprandially, differing significantly from the other treatments. In contrast, the selected strain fed either the supplemented or the non-supplemented plant-protein mixture showed almost identical patterns which were characterized by low expression of the transcript. *KLF15* is transcription factor known to contribute to the regulation of hepatic gluconeogenesis (Gray et al., 2007). Lansard et al. (2010) using rainbow trout hepatocytes demonstrated that increased available amino acids can induce an increase in expression of gluconeogenic genes with no alteration in protein synthesis. Even though no strain effect associated with alanine and aspartate transaminases was found, the interaction effect on *KLF15* expression observed in the present study and recent findings regarding plasma amino acid synchronization indicate that the selection for improved plant protein utilization might have an effect on hepatic metabolism in the selected strain. A significant interaction effect of strain by diet by time was also found on autophagy-related 4B cysteine peptidase (*ATG4b*) expression levels in muscle. The selected strain fed the plant protein mixture supplemented with crystalline amino acids showed a significant higher expression at 3 h postprandially compared to all the other treatments and consequently was decreased significantly, remaining stable and identical to its expression with the selected fish fed the unsupplemented mixture over time. In contrast the unselected strain fed either mixtures showed various peaks over time. In fish the autophagic-lysosome system has been identified as the major proteolytic system responsible for muscle protein degradation (Seiliez et al., 2014). Autophagy is mediated by several autophagy-related proteins among which Atg4 is the only gene whose activity is characterized essential and highly specific, and in rainbow trout *ATG4b* gene is considered to play a key role in muscle atrophy (Seiliez et al., 2010; Maruyama & Noda, 2018). The results of *ATG4b* transcript expression in

the present study provide further evidence that the improved growth and protein retention that the selected strain shows when fed an all-plant protein diet compared to the non-selected strain, is a result of a controlled protein digestion rate but also there is an overall different physiological homeostatic control which needs further investigation and elucidation.

5.3.1 Limitations of the Current Study

The first limitation of the current study is the same with the previous study because the same individuals were used. Thus, the sample size was less than planned which ultimately led to lower statistical power. The second major limitation is related with the number of metabolic pathways under investigation and the number of genes which are involved in each pathway. Regarding amino acid transport, the genes chosen gave a complete picture. In contrast more genes must be analyzed to more completely understand how dietary treatments affected amino acid metabolism and protein degradation pathways.

5.4 Future Research

Lately, the role of microbiota in nutrition and health of humans and animals has become a leading topic in nutritional research (Gaskins et al., 2008; Flint et al., 2012). The role of intestinal microbiota has recently been explored using a germ-free zebrafish model and microbiota were found to be involved in key processes including nutrient metabolism, innate immune response, epithelial proliferation, and overall fish growth and health (Roeselers et al., 2011). Zhao et al. (2013) suggested that host genotype and gender are among the factors influencing gut microbiota when chicken lines selected for growth were compared to unselected ones. In another study Ingerslev et al. (2014) showed that gut microbiota of rainbow trout was significantly different between fish fed plant or fishmeal-based diets. Recently, a study published by Li et al., (2013) showed a high degree of association between gut microbiota and the growth of fast-growing transgenic fish. Even though there are studies exploring the effect of SBM on the composition of gut microbiota, identification of bacterial

species has been based on culturing them in the laboratory. However, only 3-50% of gut bacterial can be grown in the laboratory (Merrifield et al., 2011), meaning that many species of gut bacteria cannot be identified using this approach. However, next generation sequencing technology overcomes this limitation and can identify the complete microbial population, allowing correlations to be made between the microbiome and fish performance (Mardis, 2008).

Traditionally, digestion is described as the process by which food in the gastrointestinal tract is split into simpler absorbable compounds performed primarily by the digestive enzymes (Ray et al., 2012). Even though, nutrient digestion in fish follows the same principles as in birds and mammals, effects of changes in diet composition and effects of biologically active compounds, such as antinutrients, may be quite different. There is relatively little mechanistic information regarding absorption and transport of dietary components such as peptides, lipids and carbohydrates in the gut. The knowledge gap in fish digestive physiology is related to: a) the regulation of digestion and absorption processes and b) the microbiota – host interactions. Research on microbiota effects on digestion has been ongoing for over a century. However only during the last two decades has the importance and basic understanding of mechanisms come to light. During this time the focus on the host-microbe interactions from the pathogen-centered perspective has neglected the fact that most microorganisms encountered by animals do not cause overt disease and can be considered as colonists that engage in commensal or mutualistic relationships with their hosts (Warnecke and Hess, 2009). The bacterial flora of the gastrointestinal tract in general represents a very important and diversified enzymatic mass that has the potential to collaboratively enhance or interfere with the metabolism of the host (Bairagi et al., 2002). There is a defined host-microbe metabolic axis that coordinates an interactive chemical communication highway between specific host cellular pathways and microbial species activities. Within these metabolic axes, multiple bacterial species can sequentially modulate distinct metabolic reactions, resulting in combinatorial metabolism of substrates by the microbiome and host, exemplified by the production of bile salts, choline, essential amino acids, vitamins and short chain

fatty acids that are essential for host health. Furthermore, the production of these metabolites by microbes not only contributes to the host metabolic phenotype but also the immunological phenotype and hence plays a significant role in disease risk (Nicholson et al., 2012).

A study by Romarheim et al., (2011) showed for the first time that a dietary inclusion of a bacterial meal in salmon diets containing high level of SBM resulted in a prevention of intestinal inflammation compared to fish fed the SBM diet without bacterial meal inclusion which showed clear signs of inflammation. The study of diseases has been classically approached from a “one nutrient-one disease” and “one microbe-one disease” viewpoint. However, just as the “one gene-one enzyme” outlook proved to be an oversimplification that failed to explain complex phenotypes, we now begin to appreciate the fact that animals like humans are colonized with a myriad of viruses and bacteria and that some disease might result from dysbiosis rather than the presence of single disease-causing microbes (Clemente et al., 2012).

Taking into consideration all the above, it is clear that future research needs to investigate the effects of genetic selection in rainbow trout on gut microbiota (composition and abundance) and the possible correlation of microbiota with the improved traits of the selected strain and the absence of intestinal inflammation.

5.5 Bibliography

Abernathy, J., A. Brezas, K. R. Snekvik, R. W. Hardy, and K. Overturf. 2017. “Integrative Functional Analyses Using Rainbow Trout Selected for Tolerance to Plant Diets Reveal Nutrigenomic Signatures for Soy Utilization without the Concurrence of Enteritis.” *PLoS ONE* 12(7).

Aldman, G., D. Grove, and S. Holmgren. 1992. “Duodenal Acidification and Intra-Arterial Injection of CCK8 Increase Gallbladder Motility in the Rainbow Trout, *Oncorhynchus mykiss*.” *General and Comparative Endocrinology* 86(1):20–25.

Ambardekar, A. A., R.C. Reigh, and M. B. Williams. 2009. “Absorption of Amino Acids from Intact Dietary Proteins and Purified Amino Acid Supplements Follows

Different Time-Courses in Channel Catfish (*Ictalurus punctatus*)." *Aquaculture* 291(3–4):179–87.

Bairagi, A., K. S. Ghosh, S. K. Sen, and A. K. Ray. 2002. "Enzyme Producing Bacterial Flora Isolated from Fish Digestive Tracts." *Aquaculture International* 10(2):109–21.

Blaufuss, P. and J. Trushenski. 2012. "Exploring Soy-Derived Alternatives to Fish Meal: Using Soy Protein Concentrate and Soy Protein Isolate in Hybrid Striped Bass Feeds." *North American Journal of Aquaculture* 74(1):8–19.

Boirie, Y., M. Dangin, P. Gachon, M. P. Vasson, J. L. Maubois, and B. Beaufrere. 1997. "Slow and Fast Dietary Proteins Differently Modulate Postprandial Protein Accretion." *Proceedings of the National Academy of Sciences* 94(26):14930–35.

van den Borne, J. J. G. C., J. W. Schrama, M. J. W. Heetkamp, M. W. A. Verstegen, and W. J. J. Gerrits. 2007. "Synchronising the Availability of Amino Acids and Glucose Increases Protein Retention in Pigs." *Animal* 1(05):666.

Bos, C., C. C. Metges, C. Gaudichon, K. J. Petzke, M. E. Pueyo, C. Morens, J. Everwand, R. Benamouzig, and D. Tomé. 2003. "Postprandial Kinetics of Dietary Amino Acids Are the Main Determinant of Their Metabolism after Soy or Milk Protein Ingestion in Humans." *The Journal of Nutrition* 133(5):1308–15.

Callet, T., F. Médale, L. Larroquet, A. Surget, P. Aguirre, T. Kerneis, L. Labbé, E. Quillet, I. Geurden, S. Skiba-Cassy, and M. Dupont-Nivet. 2017. "Successful Selection of Rainbow Trout (*Oncorhynchus mykiss*) on Their Ability to Grow with a Diet Completely Devoid of Fishmeal and Fish Oil, and Correlated Changes in Nutritional Traits." *PLoS ONE* 12(10):1–21.

Clemente, J. C., L. K. Ursell, L. Wegener Parfrey, and R. Knight. 2012. "The Impact of the Gut Microbiota on Human Health: An Integrative View." *Cell* 148(6):1258–70.

Daniel, H. 2004. "Molecular and Integrative Physiology of Intestinal Peptide Transport." *Annual Review of Physiology* 66(1):361–84.

Flint, H. J., K. P. Scott, P. Louis, and S. H. Duncan. 2012. “The Role of the Gut Microbiota in Nutrition and Health.” *Nature Reviews Gastroenterology & Hepatology* 9(10):577–89.

Gaskins, H. Rex, Jennifer A. Croix, Noriko Nakamura, and Gerardo M. Nava. 2008. “Impact of the Intestinal Microbiota on the Development of Mucosal Defense.” *Clinical Infectious Diseases* 46(s2):S80–86.

Gatlin, D. M., F. T. Barrows, P. Brown, K. Dabrowski, T. G. Gaylord, R. W. Hardy, E. Herman, G. Hu, Å. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E. J. Souza, D. Stone, R. Wilson, and E. Wurtele. 2007. “Expanding the Utilization of Sustainable Plant Products in Aquafeeds: A Review.” *Aquaculture Research* 38(6):551–79.

Gaylord, T. G., F. T. Barrows, and S. D. Rawles. 2008. “Apparent Digestibility of Gross Nutrients from Feedstuffs in Extruded Feeds for Rainbow Trout, *Oncorhynchus mykiss*.” *Journal of the World Aquaculture Society* 39(6):827–34.

Gómez-Requeni, P., M. Mingarro, J. a. Calduch-Giner, F. Médale, S. a M. Martin, D. F. Houlihan, S. Kaushik, and J. Pérez-Sánchez. 2004. “Protein Growth Performance, Amino Acid Utilisation and Somatotropic Axis Responsiveness to Fish Meal Replacement by Plant Protein Sources in Gilthead Sea Bream (Sparus aurata).” *Aquaculture* 232(1–4):493–510.

Gray, S., B. Wang, Y. Orihuela, E. Gyoung Hong, S. Fisch, S. Haldar, G. W. Cline, J. K. Kim, O. D. Peroni, B. B. Kahn, and M. K. Jain. 2007. “Regulation of Gluconeogenesis by Kruppel-like Factor 15.” *Cell Metabolism* 5(4):305–12.

Hardy, R. W. 1996. “Alternate Protein Sources for Salmon and Trout Diets.” *Animal Feed Science and Technology* 59(1–3):71–80.

Hatzoglou, M., J. Fernandez, I. Yaman, and E. Closs. 2004. “REGULATION OF CATIONIC AMINO ACID TRANSPORT: The Story of the CAT-1 Transporter.” *Annual Review of Nutrition* 24(1):377–99.

Ingerslev, H. C., L. von Gersdorff Jørgensen, M. Lenz Strube, N. Larsen, I. Dalsgaard, M. Boye, and L. Madsen. 2014. “The Development of the Gut Microbiota in

Rainbow Trout (*Oncorhynchus mykiss*) Is Affected by First Feeding and Diet Type." *Aquaculture* 424–425:24–34.

Karlsson, A., E. J. Eliason, L. T. Mydland, A. P. Farrell, and A. Kiessling. 2006. "Postprandial Changes in Plasma Free Amino Acid Levels Obtained Simultaneously from the Hepatic Portal Vein and the Dorsal Aorta in Rainbow Trout (*Oncorhynchus mykiss*)." *Journal of Experimental Biology* 209(24):4885–94.

Kaushik, S. J., J. P. Cravedi, J. P. Lalles, J. Sumpter, B. Fauconneau, and M. Laroche. 1995. "Partial or Total Replacement of Fish Meal by Soybean Protein on Growth, Protein Utilization, Potential Estrogenic or Antigenic Effects, Cholesterolemia and Flesh Quality in Rainbow Trout, *Oncorhynchus mykiss*." *Aquaculture* 133(3–4):257–74.

Krogdahl, Å., M. Penn, J. Thorsen, S. Refstie, and A. Bakke. 2010. "Important Antinutrients in Plant Feedstuffs for Aquaculture: An Update on Recent Findings Regarding Responses in Salmonids." *Aquaculture Research* 41(3):333–44.

Lansard, M., S. Panserat, E. Plagnes-Juan, I. Seiliez, and S. Skiba-Cassy. 2010. "Integration of Insulin and Amino Acid Signals That Regulate Hepatic Metabolism-Related Gene Expression in Rainbow Trout: Role of TOR." *Amino Acids* 39(3):801–10.

Larsen, B. K., J. Dalsgaard, and P. B. Pedersen. 2012. "Effects of Plant Proteins on Postprandial, Free Plasma Amino Acid Concentrations in Rainbow Trout (*Oncorhynchus mykiss*)." *Aquaculture* 326–329:90–98.

Li, X., Q. Yan, S. Xie, W. Hu, Y. Yu, and Z. Hu. 2013. "Gut Microbiota Contributes to the Growth of Fast-Growing Transgenic Common Carp (*Cyprinus carpio L.*)." *PloS One* 8(5):e64577.

Lim, S. 2004. "Effects of Dehulled Soybean Meal as a Fish Meal Replacer in Diets for Fingerling and Growing Korean Rockfish *Sebastes schlegeli*." *Aquaculture* 231(1–4):457–68.

Mambrini, M., A. J. Roem, J. P. Carvèdi, J. P. Lallès, and S. J. Kaushik. 1999. “Effects of Replacing Fish Meal with Soy Protein Concentrate and of DL-Methionine Supplementation in High-Energy, Extruded Diets on the Growth and Nutrient Utilization of Rainbow Trout, *Oncorhynchus mykiss*.” *Journal of Animal Science* 77(11):2990.

Mardis, E. R. 2008. “Next-Generation DNA Sequencing Methods.” *Annual Review of Genomics and Human Genetics* 9(1):387–402.

Martin, S. A. M., O. Vilhelmsson, F. Médale, P. Watt, S. Kaushik, and D. F. Houlihan. 2003. “Proteomic Sensitivity to Dietary Manipulations in Rainbow Trout.” *Biochimica et Biophysica Acta* 1651(1–2):17–29.

Maruyama, T. and N. N. Noda. 2018. “Autophagy-Regulating Protease Atg4: Structure, Function, Regulation and Inhibition.” *The Journal of Antibiotics* 71(1):72–78.

Merrifield, D. L., R. E. Olsen, R. Myklebust, and E. Ringø. 2011. “Dietary Effect of Soybean (*Glycine max*) Products on Gut Histology and Microbiota of Fish.” *Soy Bean and Nutrition* 231–50.

Murai, T., Ogata, H., Hirasawa, Y., Akiyama, T. & Nose, T. 1987. Portal absorption and hepatic uptake of amino acids in rainbow trout force-fed diets containing casein or crystalline amino acids. *Nippon Suisan Gakkaishi*, 53, 1847–1859.

Murashita, K., H. Fukada, I. Rønnestad, T. Kurokawa, and T. Masumoto. 2008. “Nutrient Control of Release of Pancreatic Enzymes in Yellowtail (*Seriola quinqueradiata*): Involvement of CCK and PY in the Regulatory Loop.” *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 150(4):438–43.

Naylor, R. L., R. W. Hardy, D. P. Bureau, A. Chiu, M. Elliott, A. P. Farrell, I. Forster, D. M. Gatlin, R. J. Goldburg, K. Hua, and P. D. Nichols. 2009. “Feeding Aquaculture in an Era of Finite Resources.” *Proceedings of the National Academy of Sciences of the United States of America* 106(36):15103–10.

Nicholson, J. K., E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, and S. Pettersson. 2012. “Host-Gut Microbiota Metabolic Interactions.” *Science* 336(6086):1262–67.

National Research Council (NRC). 2011. Nutrient Requirements of Fish and Shrimp. *National Academic Press*, Washington, DC.

Olli, J. J. and Å. Krogdahi. 1994. “Nutritive Value of Four Soybean Products as Protein Sources in Diets for Rainbow Trout (*Oncorhynchus mykiss* , Walbaum) Reared in Fresh Water.” *Acta Agriculturae Scandinavica, Section A - Animal Science* 44(3):185–92.

Orozco, Z. A. Gaye, S. Soma, T. Kaneko, and S. Watanabe. 2018. “Spatial MRNA Expression and Response to Fasting and Refeeding of Neutral Amino Acid Transporters Slc6a18 and Slc6a19a in the Intestinal Epithelium of Mozambique Tilapia.” *Frontiers in Physiology* 9:212.

Overturf, K., F. T. Barrows, and R. W. Hardy. 2013. “Effect and Interaction of Rainbow Trout Strain (*Oncorhynchus mykiss*) and Diet Type on Growth and Nutrient Retention.” *Aquaculture Research* 44(4):604–11.

Ray, A. K., K. Ghosh, and E. Ringø. 2012. “Enzyme-Producing Bacteria Isolated from Fish Gut: A Review.” *Aquaculture Nutrition* 18(5):465–92.

Refstie, S., O. J. Korsoen, T. Storebakken, G. Baeverfjord, I. Lein, and a. J. Roem. 2000. “Differing Nutritional Responses to Dietary Soybean Meal in Rainbow Trout (*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*).” *Aquaculture* 190(1–2):49–63.

Roeselers, G., E. K. Mittge, W. Z.Stephens, D.M. Parichy, C. M. Cavanaugh, K. Guillemin, and J. F. Rawls. 2011. “Evidence for a Core Gut Microbiota in the Zebrafish.” *The ISME Journal* 5(10):1595–1608.

Rolland, M., J. Dalsgaard, J. Holm, P. Gómez-Requeni, and P. V. Skov. 2015. “Dietary Methionine Level Affects Growth Performance and Hepatic Gene Expression of GH-IGF System and Protein Turnover Regulators in Rainbow Trout (*Oncorhynchus mykiss*) Fed Plant Protein-Based Diets.” *Comparative*

Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology 181:33–41.

Rolland, M., B. K. Larsen, J. Holm, J. Dalsgaard, and P. V. Skov. 2015. “Effect of Plant Proteins and Crystalline Amino Acid Supplementation on Postprandial Plasma Amino Acid Profiles and Metabolic Response in Rainbow Trout (*Oncorhynchus mykiss*).” *Aquaculture International* 23(4):1071–87.

Romarheim, O. H., M. Øverland, L. T. Mydland, A. Skrede, and T. Landsverk. 2011. “Bacteria Grown on Natural Gas Prevent Soybean Meal-Induced Enteritis in Atlantic Salmon.” *The Journal of Nutrition* 141(1):124–30.

Schuhmacher, A., J. Schön, M. Goldberg, and J. M. Gropp. 1995. “Plasma Amino Acid Levels in Rainbow Trout (*Oncorhynchus mykiss*).” *Journal of Applied Ichthyology* 11(3–4):309–16.

Schuhmacher, A., C. Wax, and J. M. Gropp. 1997. “Plasma Amino Acids in Rainbow Trout Fed Intact Protein or a Crystalline Amino Acid Diet.Pdf.” 151:15–28.

Seiliez, I., K. Dias, and B. M. Cleveland. 2014. “Contribution of the Autophagy-Lysosomal and Ubiquitin-Proteasomal Proteolytic Systems to Total Proteolysis in Rainbow Trout (*Oncorhynchus mykiss*) Myotubes.” *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 307(11):R1330–37.

Seiliez, I., J. Gutierrez, C. Salmerón, S. Skiba-Cassy, C. Chauvin, K. Dias, S. Kaushik, S. Tesseraud, and S. Panserat. 2010. “An in Vivo and in Vitro Assessment of Autophagy-Related Gene Expression in Muscle of Rainbow Trout (*Oncorhynchus mykiss*).” *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 157(3):258–66.

Seiliez, I., S. Panserat, S. Skiba-Cassy, A. Fricot, C. Vachot, S. Kaushik, and S. Tesseraud. 2008. “Feeding Status Regulates the Polyubiquitination Step of the Ubiquitin-Proteasome-Dependent Proteolysis in Rainbow Trout (*Oncorhynchus mykiss*) Muscle.” *The Journal of Nutrition* 138(3):487–91.

Stickney, R. R., R. W. Hardy, K. Koch, R. Harrold, D. Seawright, and K. C. Massee. 1996. “The Effects of Substituting Selected Oilseed Protein Concentrates for Fish Meal in Rainbow Trout *Oncorhynchus mykiss* Diets.” *Journal of the World Aquaculture Society* 27(1):57–63.

Walker, D., D. T. Thwaites, N. L. Simmons, H. J. Gilbert, and B. H. Hirst. 1998. “Substrate Upregulation of the Human Small Intestinal Peptide Transporter, HPepT1.” *The Journal of Physiology* 507(3):697–706.

Warnecke, F. and M. Hess. 2009. “A Perspective: Metatranscriptomics as a Tool for the Discovery of Novel Biocatalysts.” *Journal of Biotechnology* 142(1):91–95.

Wu, L., L. He, Z. Cui, G. Liu, K.Yao, F. Wu, J. Li, and T. Li. 2015. “Effects of Reducing Dietary Protein on the Expression of Nutrition Sensing Genes (Amino Acid Transporters) in Weaned Piglets.” *Journal of Zhejiang University. Science. B* 16(6):496–502.

Yamamoto, T., T. Unuma, T. Akiyama. 1998. Postprandial changes in plasma free amino acid concentrations of rainbow trout fed diets containing different protein sources. *Fish. Sci.* 64, 474–481.

Zhao, L., G. Wang, P.Siegel, C. He, H. Wang, W. Zhao, Z. Zhai, F. Tian, J. Zhao, H. Zhang, Z. Sun, W. Chen, Y. Zhang, and H. Meng. 2013. “Quantitative Genetic Background of the Host Influences Gut Microbiomes in Chickens.” *Scientific Reports* 3:1–6.