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THE PRACTICAL APPLICATION OF IN-VITRO TISSUE DIGESTION AS A MEANS OF PRODUCING SPECIES-SPECIFIC LARVAL DIETS AND THE IMPACT OF DIETARY PROTEIN COMPOSITION ON GROWTH AND METABOLISM IN FRESHWATER FISH

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by

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DISSERTATION APPROVAL

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for the Degree of

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in the field of Zoology

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MAJOR PROFESSOR: Dr. Karolina Kwasek

The heavy reliance on live feeds is currently restricting the growth and sustainability of the aquaculture industry, therefore, the overall goal of this research was to improve the utilization of formulated dry diets at first feeding of larval fish. This was done with a specific focus on the production and provision of the optimal dietary protein form and composition.

Chapter 2 aimed to provide an efficient protein source for larval fish by using same-species muscle and endogenous enzymes to produce hydrolysates and by providing a series of diets with increasing molecular weight protein fragments through larval development. Largemouth Bass (*Micropterus salmoides*) (LMB) muscle was mixed with the digestive enzymes from adult LMB and hydrolyzed for 1.5, 3, and 6 h, respectively. Five diets were produced, an intact diet containing non-hydrolyzed muscle and four diets with 37% muscle hydrolysate inclusion. The molecular weight profile of those diets were formulated to vary based on the inclusion level of each hydrolysate. To account for gut development, one group of larval LMB was fed a weekly series of diets with an increasing molecular weight profile. The initial inclusion of the hydrolysates significantly improved the total length of the larval LMB; however, neither the hydrolysate inclusion nor the series of dietary molecular weight profiles improved the overall growth of larval LMB. The inclusion of hydrolysates significantly decreased the occurrence of skeletal deformities. The results from this study suggest that the inclusion of same-species

hydrolysates can improve the initial growth of first-feeding LMB, but further research is necessary to determine the optimal molecular weight profile, hydrolysate inclusion level, and physical properties of feeds to improve the overall growth performance during the larval stage.

Chapter 3 compared the effect of dietary inclusion of a fish muscle hydrolysate produced from species-specific muscle and enzymes to hydrolysates produced from those of a different species, in diets for larval Walleye (*Sander vitreus*). Four intact and hydrolyzed protein products were produced from each combination of Walleye muscle and endogenous enzymes, and muscle and endogenous enzymes from Nile Tilapia (*Oreochromis niloticus*). The hydrolyzed products were continuously mixed for 3 h during the hydrolysis, (at 22°C and 28°C for Walleye and Tilapia enzymes, respectively), and the pH was adjusted throughout the process to mimic gastric and intestinal digestion conditions. Four diets were produced with the dietary protein supplied as a 50/50 ratio of the intact and hydrolyzed muscle from the respective muscle/enzyme combination. There was a significant interaction effect between muscle and enzyme source on the growth of larval Walleye. At the conclusion of the study, the larval Walleye that received the diet with muscle hydrolysate produced with Walleye muscle and Walleye endogenous enzymes had a significantly higher average weight than all other groups, and significantly higher postprandial levels of total free amino acids and indispensable amino acids in the muscle. Each hydrolysate-based diet led to a significant reduction in skeletal deformities and survival, compared to a group fed with a commercial diet. The results from this study suggest that species-specific muscle and enzymes produce a more optimal dietary protein source for larval fish than non-species-specific products, but further research should focus on improving the physical properties of the formulated diets to improve survival of fish larvae.

Chapter 4 proposed a practical controlled hydrolysis method to utilize the endogenous enzymes within the fish body for the breakdown of tissues proteins, and to produce a species-specific meal that is tailored to the nutritional requirements and absorptive capacity of fish larvae. Four Zebrafish (*Danio rerio*) meals were produced from whole-body adult Zebrafish, three hydrolysates that were hydrolyzed for 1, 2, and 3 h, respectively, and an unhydrolyzed meal. From these meals, three diets were produced, each defined by their supply of dietary protein. The Unhydro diet was solely based on the unhydrolyzed Zebrafish meal. The 50% Hydro diet was based on 50% Zebrafish hydrolysate mix and 50% unhydrolyzed Zebrafish meal. The 100% Hydro diet was 100% based on the Zebrafish meal hydrolysate. The hydrolysate mix contained equal parts of the 1, 2, and 3 h hydrolysates. Proteomic analysis showed that the proposed hydrolysis method was able to efficiently hydrolyze the protein within Zebrafish body. The feeding trial found no significant differences in the final weight, total length, or survival between the Unhydro, 50% Hydro, and 100% Hydro groups, but the 50% Hydro group did express a significant upregulation of PepT1 at 24 h after feeding, compared to the Unhydro group. The growth results paired with PepT1 gene expression potentially indicate Zebrafish larvae to be adapted to dry feeds at first feeding and able to utilize dietary protein in different molecular forms efficiently for growth. Overall, the proposed hydrolysis method provides a practical and cost-effective approach to producing species-specific fishmeal hydrolysates. Further research is necessary to determine whether the produced hydrolysates can improve the growth of larval fish in other fish models.

Further insight into behavioral and physiological responses in fish to imbalanced dietary amino acid profiles was provided in Chapter 5. The objective of this study was to determine how stomachless fish respond to diets deficient in the main limiting IDAA (lysine, methionine, and

threonine), using Zebrafish as a model species. Six semi-purified diets were formulated for this study. The CG diet contained casein and gelatin as its only protein sources, while FAA50 diet had 50% of is dietary protein supplied with crystalline amino acids. Both were formulated to contain identical, balanced amino acid profiles. The remaining diets were supplied with the same amino acid mix as the FAA50 diet, but with minor adjustments to create deficiencies of the selected IDAA. The (-) Lys, (-) Met, and (-) Thr diets had lysine, methionine, and threonine withheld from the free amino acid (FAA) mix, respectively, and the Def diet was deficient in all three. The fish were fed to apparent satiation three times a day, and each feeding was carefully observed to ensure all feed added to the tanks was consumed. The results showed that although the singular deficiency of the three main limiting amino acids did not induce significant changes in feed intake, the combined deficiency of the three IDAA significantly increased the feed intake of juvenile Zebrafish. This increased feed intake prevented the IDAA deficiencies from significantly reducing growth, however, the feeding efficiency was also reduced. There was also an observed upregulation of *neuropeptide Y* (*NPY*), an orexigenic hormone, in the Def group, compared to the FAA50 group. The outcomes of this study provide insight into the behavioral and physiological response to dietary amino acid imbalances of stomachless fish and suggests stomachless fish increase their feed intake when challenged with IDAA-deficient diets, and that the regulation of *NPY* might play a role in this response.

Chapter 6 assessed the postprandial FAA dynamics in the plasma, liver, and muscle of three species; 1) Largemouth Bass – warm-water, stomach-possessing carnivorous species; 2) Walleye – cool-water, stomach-possessing carnivorous species; and 3) Zebrafish– tropical, stomachless omnivorous species. Two diets were formulated for this study, a diet based on intact casein and gelatin (CG), and a diet with 50% of its protein supplied in FAA form (FAA50).

Forty-two fish from each species were utilized, with one group of 21 receiving the CG diet, and the other 21 receiving the FAA50 diet. All fish were starved for 24 hours prior to the final feeding before sampling. Three fish were sampled at each time point, with three samples (plasma, liver, and muscle) taken from each fish. Samples were taken prior to feeding (0 h) and then at 0.5, 1, 2, 3, 6, and 12 h after feeding, for all species. A significant three-way interaction was observed between the diet, species, and postprandial time on the total FAA, IDAA, and DAA levels in the plasma, liver, and muscle, indicating that the postprandial FAA patterns were significantly different between species and in response to the different diets. In stomach-possessing species, dietary amino acids from the FAA50 diet were absorbed more rapidly than those from the CG diet, resulting in fewer correlations with the dietary IDAA profiles. The absorption of FAA in cool-water Walleye was more gradual and prolonged than the warm-water LMB, leading to more significant correlations with the dietary IDAA and more sustained peaks. The postprandial peaks of FAA typically occurred at the same time in the stomachless Zebrafish fed with the CG or FAA50 diet. The levels of FAA were noticeably lower after feeding with the FAA50 diet in Zebrafish, compared to the CG diet. These results provide a reference for differences in the FAA dynamic patterns of three species with differing physiological characteristics, when fed diets with intact protein or supplemented with FAA.

The findings presented in this dissertation provide support and novel methods for the production and inclusion of species-specific protein hydrolysates as an ideal protein source in formulated diets for first-feeding larval fish. This research contributes to the development of larval diets that can release the limitations of growth placed on the aquaculture industry by the reliance on live feeds, particularly within the hatchery sector. This research also provides further

understanding of dietary protein utilization and delivers new fish nutrition knowledge that will benefit the aquaculture industry as a whole.

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DEDICATION

To my parents, for their love, support, and guidance that made this all possible.

PREFACE

The content provided in Chapter 2 has been published prior to the submission of this dissertation:

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CHAPTER 1

INTRODUCTION

Over the past three decades, the harvest of wild-caught fish has plateaued. This plateau is a result of overfishing and significant pressure applied on wild stocks to sustain the increased demand for aquatic animal protein of a growing world human population. Thus, to meet the increasing demand, aquaculture production has significantly increased within that same timeframe (FAO, 2022). Aquaculture is the controlled rearing of aquatic organisms for human consumption or stocking purposes (Tacon and Metian, 2015). Aquaculture currently contributes more than half of the total worldwide production of aquatic animals, and 83% of total harvest from freshwaters (FAO, 2022). By 2030, aquaculture is expected to contribute over 60% of the total aquatic harvest (Kibenge, 2016). The achievement of this projection is a result of decades of intensive research to address certain challenges that present bottlenecks to the future growth of aquaculture. One of these challenges facing the industry is the unstable production of high-quality juveniles and adults of many species (Conceição and Tandler, 2018) that hinges on successful and efficient larval rearing. Unfortunately, current larval rearing methods are often characterized by uncontrolled environmental factors, and highly variable, inefficient nutritional inputs (Conceição and Tandler, 2018).

During the larval stage, fish rely on high levels of amino acids for the synthesis of new tissues, and to provide energy throughout this period of significant physiological and morphological change (Parra et al. 1999; Rønnestad et al. 2003). Thus, the proper selection of dietary protein and delivery of amino acids is imperative to support the appropriate development and high growth potential of larval fish. Current commercial rearing protocols heavily rely on live feeds to deliver these amino acids, especially at the onset of exogenous feeding (Qin, 2008;

Holt, 2011; Hamre et al. 2013). Common live feed organisms include algae, rotifers, copepods, *Artemia* nauplii, and many others that are readily ingested by larvae. These free-swimming organisms offer prolonged availability in the water column (Rasdi and Qin, 2016; Samat et al. 2020), and increased detectability due to quick, erratic movements (Øie et al. 2011; Samat et al. 2020). Live feeds are especially beneficial to larval fish with underdeveloped digestive tracts, as they contain water-soluble nutrients that are easily digestible and contribute enzymes that help autolyze their own tissue after ingestion (Kolkovski, 2001; Langer et al. 2009). Due to these benefits, larval rearing often takes place in outdoor nursery ponds that are fertilized to promote high densities of live feed organisms. The use of these nursery ponds can result in unpredictable production levels, mostly due to the lack of control over environmental factors that can affect larval growth and survival. Given the need for the more efficient, predictable production of high-quality juveniles to promote sustainable growth of the aquaculture industry, there is a push to further intensify commercial larval rearing practices (Little et al. 2016).

Live feeds require a separate culture within an intensive aquaculture operation, adding labor intensive maintenance and additional costs (Ehrlich et al. 1989; People Le Ruyet et al. 1993; Kolkovski, 2001; Cahu and Infante, 2001; Callan et al. 2003; Fletcher et al. 2007; Holt, 2011). The separate culture of live feed also presents biosecurity risks. The biosecurity risks stem from the ability for live feed organisms to serve as a vector for pathogens. The live feed organisms utilized in larval rearing are usually filter-feeders, absorbing nutrients from the water without selectivity (Øie et al. 2011). Therefore, a risk that pathogens will be transmitted along the food chain and into larval rearing systems is high (Su et al. 2005; Petersen et al. 2013; Watts et al. 2016). Additionally, the market availability of certain live feeds can fluctuate greatly based

on environmental and harvest conditions (i.e., Salt Lake strain of *Artemia*), which adds the unpredictability of an inconsistent supply-chain for larval feeds (Kolkovski, 2001).

The heavy reliance on live feeds also presents nutritional disadvantages for larval rearing. The overall nutritional profiles of live feed are highly variable, and commonly lack sufficient levels of essential nutrients (Conceição et al. 2010; Kandathil Radhakrishna et al. 2020). They also possess a high-water content (~80%), which reduces their efficiency of nutrient delivery (Conceição et al. 2010). This also means that high amounts of live feed organisms need to be consumed to fulfill the nutrient requirements of the larval fish.

These disadvantages to the use of live feed currently serve as a major roadblock to the effective larval rearing of many current commercial species, and the potential intensive rearing of new species. Thus, research has focused on the production of dry diets to replace live feeds.

Dry diets are formulated feeds designed to meet nutritional requirements of fish to maximize their growth performance and survival. Those feeds are agglomerated or extruded, dried, easily stored, and presented to the fish in either a pellet, flakes, crumble, or microparticulate form. Live feed replacement with these dry diets would provide a more optimal and consistent nutritional input for larvae, reduce labor and feed costs, and ensure constant availability of larval feeds (Holt, 2011; Jobling, 2016; Lipscomb et al. 2020). The full switch to dry diets would also assist in the more stable and predictable production of larval fish by increasing the potential for intensive rearing of more species. Improvements have been made towards earlier complete weaning onto dry diets for larval fish (Holt, 1993; Curnow et al. 2006; Sheng et al. 2022), however successful results on the complete replacement of live feed with dry diets at first feeding are scarce. The full replacement of live feed has led to poor feed intake and significant reductions in survival and growth performance of fish larvae of many species

(Walford et al. 1991; People Le Ruyet et al. 1993; Canavate and Fernández-Diaz, 1999; Cahu et al. 1998; Goolish et al. 1999; Lazo et al. 2000; Fletcher et al. 2007; Sarvi et al. 2009; Adekunle and Joyce, 2014). In addition to the stunted growth, larval fish fed with a dry diet have shown a significant increase in skeletal deformities, which has a negative impact on the production levels of hatcheries, reducing the quality and survival of the reared fish (Cahu et al. 2003; Boursiaki et al. 2019).

One of the key characteristics of a proper formulated diet for larval fish includes prolonged nutrient retention in water, ease of ingestion, and high digestibility (Hamre et al. 2013). The typical dietary protein sources included in aquaculture diets are animal-based meals (typically fish meal) and plant meals (NRC, 2011). These consist of complex, intact proteins that are not efficiently digested and absorbed by larval fish (Holt, 2011; Rønnestad et al. 2013). Many commercial species are altricial species, characterized by a short, underdeveloped gut and reduced enzymatic activity compared to adults (Kolkovski, 2001; Cahu and Infante, 2001; Rønnestad et al. 2013). Altricial larvae do not possess a differentiated and functioning stomach among other organs that belong to the digestive system at hatching, which results in the lack of pepsin activity, a gastric proteolytic enzyme that functions to break down complex proteins in stomach-possessing juveniles and adults (Holt, 2011). This hinders their ability to efficiently breakdown complex proteins present in fish meals and reduces their absorption of dietary amino acids from these diets (Lazo et al. 2011). Additionally, the breakdown of these complex proteins is an extensive process and due to the short retention time of diets in the gut, the evacuation levels of undigested protein increase with the complexity of protein (D'Abramo, 2002; Lazo et al. 2011). To account for this reduced digestive capacity and short gut retention time, the

inclusion of smaller protein fragments has been studied as a mean of improving the larval digestion and absorption of dietary amino acids.

The provision of these small protein fragments has typically come from the dietary inclusion of protein hydrolysates. Protein hydrolysates are complex proteins that have undergone facilitated hydrolysis to produce high proportions of oligopeptides, small peptides, and free amino acids (Cahu and Infante, 2001). The protein source, enzymes source, and degree of hydrolysis all contribute to the amino acid and molecular weight profile of the protein hydrolysate, with a higher degree of hydrolysis creating smaller protein fragments (Kotzamanis et al. 2007). Protein hydrolysates also serve as a dietary palatability enhancer, which increases feed intake of the pelleted diets (Hamre et al. 2013). Although results on the effects of dietary inclusion of protein hydrolysates vary, there is a general consensus that moderate replacement of intact protein with hydrolyzed protein significantly improves the performance of larval fish fed with dry diets (Carvahlo et al. 1997; Cahu and Infante, 2001; Srichanun et al. 2014; Delcroix et al. 2015; Canada et al. 2017; Kwasek et al. 2021; Printzi et al. 2023). However, even with these successes, the full replacement of live feeds is not yet possible for most commercial species (Holt, 2011; Rathore et al. 2016).

Not only must the diet be ingested and efficiently digested by the larvae, it also needs to meet the specific nutrient requirements that vary between species. The nutritional requirements of larval fish are significantly different from those of adults and juveniles and are difficult to quantify with current methods (NRC, 2011). Given the importance of dietary amino acids to the proper growth and development of larvae, ensuring the species-specific amino acid requirements are met with dry diets is critical. Therefore, it was important to provide insight into novel ways of meeting these species-specific amino acid requirements and improve upon the previously

observed successes of dietary protein hydrolysates to ensure proper absorption and utilization of the provided amino acids. Addressing this need would further promote the production of a dry diet suitable to sustain high levels of growth and survival in larval fish without the need for live feeds.

Experimental Species

Largemouth Bass (LMB) is a warm-water carnivorous, stomach-possessing species. It is a top sport fishing species, produced for stocking into fisheries, and is also important in live markets in both the United States and Asia (Tidwell et al. 2003). Largemouth Bass is an altricial stomach-possessing species, characterized by an underdeveloped larval digestive tract that is unable to efficiently utilize and absorb the complex proteins within formulated diets (Skudlarek et al. 2013). This has resulted in significant reductions in survival when larval LMB are fed with a formulated diet immediately after mouth opening (Skudlarek et al. 2013). Due to this, LMB are typically raised in fertilized ponds with access to live feed organisms. This type of production can be inconsistent due to variables like temperature and live feed availability having significant impact on survival and causing fluctuating production numbers each season. Thus, developing a formulated diet for larval LMB is key in improving production efficiency of LMB fingerlings and reducing costs associated with fluctuating survivability.

Similar to LMB, Walleye (*Sander vitreus*) is a popular sport fish that is commonly raised for stocking purposes, however, the production of Walleye as food fish is still limited. Walleye is a cool-water carnivorous and stomach-possessing fish, but their optimal temperature for growth limits the production season and makes pond culture difficult (Johnson et al. 2008).

Walleye is an altricial species and, although they have shown some acceptance for formulated feeds (Johnson et al. 2008), an optimal larval diet for this species has not been

developed yet. This means larval production is still predominantly carried out in ponds with the use of live feed (Johnson et al. 2008). The production of a dry diet that can support optimal growth and survival during the larval stage would increase the potential for more intensive rearing of Walleye.

The last species to be investigated was Zebrafish (*Danio rerio*), a tropical, omnivorous, stomachless species. Zebrafish is a popular model organism for a wide variety of research areas such as nutrition, physiology, biomedical research, genetics, etc. (Ribas and Pifferer, 2014; Ulloa et al. 2014). Zebrafish can serve as a strong model species for testing nutritional approaches for commercial species (Ribas and Pifferer; 2014). Additionally, Zebrafish provided a reference for the use of our novel approaches in stomachless species, which presents contrasts in dietary protein digestion processes and overall digestive physiology from stomach-possessing species.

Main Goal

Given the constraints placed on the aquaculture industry by the heavy reliance on live feeds, the overall goal of this research was to improve the utilization of formulated dry diets at first feeding of larval fish. This was done with a specific focus on the production and provision of the optimal dietary protein form and composition. Five studies were conducted to achieve this goal. Chapter 2 sought to identify an ideal feeding regime and protein hydrolysate profile for larval Largemouth Bass as they metamorphose into juveniles; Chapter 3 investigated the protein and enzyme sources for obtaining optimal protein hydrolysates for larval diets for Walleye; and Chapter 4 tested a novel approach to produce species-specific fish meal hydrolysates as a dietary protein source for larval Zebrafish. Additional insight was also provided to better understand the utilization of dietary protein/amino acids in Chapter 5, where the behavioral and physiological response to dietary amino acid imbalances were assessed in Zebrafish; and Chapter 6 where the

postprandial free amino acid dynamics were measured in response to different molecular weight profiles of dietary protein in Largemouth Bass, Walleye, and Zebrafish. The specific introductions in each chapter provide a more detailed explanation of each approach taken.

CHAPTER 2

A NOVEL APPROACH IN THE DEVELOPMENT OF LARVAL LARGEMOUTH BASS *MICROPTERUS SALMOIDES* DIETS USING LARGEMOUTH BASS MUSCLE HYDROLYSATES AS THE PROTEIN SOURCE

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ABSTRACT

This study aimed to provide an efficient protein source for larval fish by using same-species muscle and endogenous enzymes to produce hydrolysates and by providing a series of diets with increasing molecular weight protein fragments through larval development. The specific objectives were to determine the effect of Largemouth Bass (*Micropterus salmoides*) (LMB) muscle hydrolysates obtained using same-species digestive enzymes and the degree of LMB muscle hydrolysis when included in the first feeds on growth performance and survival, skeletal development, intestinal peptide uptake, and muscle-free amino acid composition of larval LMB. LMB muscle was mixed with digestive enzymes from adult LMB, and hydrolyzed for 1.5, 3, and 6 h, respectively. Five diets were produced, the intact diet containing non-hydrolyzed muscle and four diets with 37% muscle hydrolysate inclusion. Those diets were characterized by their level of each hydrolysate (presented as a ratio of 1.5, 3, and 6 hydrolysates): 1:1:1, 1:3:6, 1:3:1, 6:3:1 for diets A, B, C, and D, respectively. To account for gut development, one group of larval LMB was fed a weekly series of diets B, C, and D to provide an increasing molecular weight profile throughout development. This group was compared against others that received either; (1) diets D, C, and B; (2) diet A; or (3) intact diet. The initial

inclusion of the hydrolysates significantly improved the total length of the larval LMB; however, neither the hydrolysate inclusion nor the series of dietary molecular weight profiles improved the overall growth of larval LMB. The inclusion of hydrolysates significantly decreased the occurrence of skeletal deformities. The degree of hydrolysis did not have a significant effect on the parameters measured, except for intestinal peptide uptake, which was increased in the group that received the most hydrolyzed diet at the final time of sampling. The lack of overall growth improvement suggests that while the hydrolysates improve the initial growth performance, further research is necessary to determine the optimal molecular weight profile, hydrolysate inclusion level, and physical properties of feeds for larval LMB.

INTRODUCTION

Currently, the successful larval rearing of many marine and freshwater species relies heavily on the live feed, such as algae, rotifers, copepods, and *Artemia* nauplii. Live feed provides significant advantages, such as prolonged availability in the water column and the ability to trigger the natural feeding response of larvae through quick, jerky movements (Samat et al. 2020). However, this heavy reliance on live feed poses major challenges to the aquaculture industry. Live feed is much more labor-intensive and expensive than dry-formulated diets (Cahu and Infante, 2001; Callen et al. 2003; Fletcher et al. 2007) and the nutritional profiles of live feed are inconsistent and may lack essential nutrients (Conceição et al. 2010; Kandathil Radhakrishnan et al. 2020). Thus, to alleviate the reliance on the live feed, research has focused on developing optimal formulated diets for larval fish able to sustain high growth rates and survival.

Typically, replacing live feed with dry diets has resulted in significantly reduced growth and survival in both marine (Walford et al. 1991; People Le Ruyet et al. 1993; Lazo et al. 2000;

Fletcher et al. 2007; Sarvi et al. 2009; Khoa et al. 2020; Mozanzadeh et al. 2021) and freshwater species (Goolish et al. 1999; Adekunle and Joyce, 2014; Jamali and Meshkini, 2018; Lipscomb et al. 2020). Additionally, skeletal deformities, including scoliosis and lordosis, have increased significantly in larval fish fed with a dry diet (Cahu et al. 2003). The hindered performance of larval fish on dry feed has been attributed to two major factors. Firstly, the nutritional requirements of larval fish are difficult to quantify. To estimate the optimal amino acid profile for the larval fish, analysis of the amino acid profile of the adult fish has been suggested (Mambrini and Kaushik, 1995; Akiyama et al. 1997; Meyer and Fracalossi, 2005). This concept is based on the "Ideal Protein" (Cole, 1980), which assumes that the optimal amino acid requirements of a fish are reflected by its whole-body amino acid profile (Wilson and Cowey 1985). This suggests that the muscle of the adult fish contains the optimal amino acid composition required by its larval stage and, hence, the adult stage muscle fulfills requirements for the exact amino acid profile required for larval growth and development. Secondly, in larval fish, the capacity to digest protein is reduced and the larvae are not able to efficiently break down and absorb intact dietary proteins (Dabrowski, 1984; Kolkovski, 2001). To combat this, protein hydrolysates have been suggested as an optimal protein source for larval fish (Zambonino Infante at al. 1997). The inclusion of protein hydrolysates in formulated dry diets has been tested previously and positive results on growth performance and the occurrence of skeletal deformities have been observed at dietary inclusion levels lower than 50% (Cahu et al. 2003; Zambonino Infante et al. 1997; Fernández et al. 2008; Kwasek et al. 2021). In addition to inclusion level, the protein sources used, conditions (pH, temperature), and the enzymes utilized are all critical in successful protein hydrolysate production and utilization (Kotzamanis et al 2007). All of these factors can determine not only the protein profile of the hydrolysate, but also its palatability and

proportion of polypeptides, oligopeptides, free amino acids (FAA), etc. (Swanepoel and Goosen, 2018), which can significantly impact how efficiently larval fish utilize hydrolysate-based diets (Kotzamanis et al. 2007; Srichanun et al. 2014). Kwasek et al. (2021) used Bighead Carp (*Hypophthalmichthys nobilis*) muscle as a protein source in the diets of larval Largemouth Bass (*Micropterus salmoides*) (LMB). The carp muscle was hydrolyzed with the endogenous enzymes from adult LMB in vitro and its dietary inclusion improved growth performance and reduced skeletal deformities of the larval LMB (Kwasek et al. 2021). However, the live feed could not be fully replaced using the carp hydrolysate-based diet, possibly suggesting that the protein source used for larval LMB diets should more closely match its nutritional requirements.

Another consideration in developing an optimal formulated dry diet for larval fish is how the digestive tract changes throughout development during the larval stage. As the digestive tract matures, a shift in enzymatic activity occurs from higher levels of peptidases designed to break down smaller peptides, towards increased levels of enzymes, such as pepsin (gastric species) and trypsin (gastric and agastric species), that function to break down intact proteins into smaller peptides (Dabrowski, 1984; Kolkovski, 2001). Based on this, the optimal protein sizes for larval fish increase as the fish metamorphose and the digestive tract develops (Canada et al. 2017). Canada et al. (2017) observed that pre-metamorphic Senegalese Sole (*Solea senegalensis*) utilized lower-molecular-weight peptides (5–70 kDa) better than larger, intact proteins. In comparison, post-metamorphic Sole absorbed the intact protein more efficiently than the lower-molecular-weight fractions. Thus, providing larval fish with the right proportion of FAA and/or di-/tripeptides and then shifting to larger polypeptides and or intact protein as metamorphosis continues seems to present an ideal feeding regimen for optimal larval development.

To address these challenges and develop an optimal formulated dry diet and feeding regimen for larval LMB, this study had two objectives; (1) to evaluate the utilization of same-species muscle hydrolyzed with same-species digestive enzymes as a protein source in the diets for larval LMB; and (2) to investigate how the molecular weight profile of the dietary protein affected utilization throughout larval development. These objectives were evaluated based on growth performance and survival, the occurrence of skeletal deformities, the expression of intestinal peptide transporter PepT1, and postprandial muscle FAA composition used as an indicator of dietary amino acid availability.

MATERIALS AND METHODS

Experimental Conditions

The feeding trial was conducted at the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University-Carbondale (SIUC), IL. All experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of SIUC. The SIUC Institutional Animal Care and Use approved all of the protocols performed (protocol #18–051). The experiment was carried out using a semi-recirculated aquaculture system with two mechanical (sand) filters (Pentair, Minneapolis, MN, USA) and a bio-filter. The system consisted of 30 (280 L) black tanks. The photoperiod was set to 14 h of darkness and 10 h of light, with the overhead lights on from 8 a.m. to 6 p.m. The system was supplied with groundwater, with a constant inflow of 100 mL/min into each tank. During the study, the temperature was 24.72 °C (±0.79) and the pH was 8.45 (±0.08). The salinity of the system was kept at 1–3 ppt in order to prolong the viability of the live feed (Dabrowski and Miller, 2018).

Muscle Hydrolysis

The hydrolysis method for this study was based on the in vitro method described by Kwasek et al. (2021) with some modification. Adult LMB (2-year-old males and females) were kept at 25 °C and fed to satiation (one meal). Approximately 2 h after feeding, the fish were euthanized with an excess of tricaine methanesulfonate (MS-222) (#E10521, Sigma-Aldrich, St. Louis, MO, USA) with a dose of 0.4 mg/mL. The head and tail of each fish were removed, and their digestive tracts were dissected and placed on ice. The remaining carcass was ground three times with a meat grinder (General Food Service, Weston, FL, USA), diluted with deionized water (1:2), and homogenized with a PowerGen 1000 (Fisher Scientific, Waltham, MA, USA) tissue homogenizer on high speed for 10 min. The digestive tracts were also diluted with deionized water (1:2), homogenized for 10 min, and the homogenates were centrifuged (1500× *g* for 10 min at 4 °C) to obtain the supernatant and to separate it from the solid mass (including undigested feed, fat, and tissues). The supernatant was then immediately used for muscle hydrolysis.

The LMB muscle homogenates were moved to containers (12 L), placed in a water bath (25 °C), diluted with deionized water (1:2), and stirred using an overhead stirrer (VWR VOS 16, VWR, Radnor, PA, USA) for the duration of the hydrolysis. These muscle homogenates were mixed with the digestive tract supernatant (3:1 muscle:supernatant). This ratio was utilized to match the amount of protein one LMB digestive tract consumes during one meal when fed at a feeding rate of 1% body weight with a commercial diet. Part of the homogenate and supernatant mixture was not incubated and was brought to 90 °C for 15 min immediately after mixing to stop the enzymatic activity. This sample served as the intact protein source (control). Since the intact muscle was exposed to enzymes for a limited time, it cannot be treated as completely

unhydrolyzed tissue. However, it did not undergo the specific controlled hydrolysis process and incubation in different pH conditions. Therefore, it is referred to as intact in this study in order to clearly differentiate it from the muscle that was incubated and underwent the hydrolysis process. The rest of the mixture underwent hydrolysis and was kept at 25 °C for the entire process to match the holding temperature of the bass. The mixture was kept at a pH of 3–4 to mimic stomach digestion and then at a pH of 7–9 for the remainder of the hydrolysis to mimic intestinal digestion. Three different hydrolysates were obtained:

- **1.5 h**: 1 h at pH 3–4, followed by 30 min at pH 7–9
- **3 h**: 1 h at pH 3–4, followed by 2 h at pH 7–9
- **6 h**: 1 h at pH 3–4, followed by 5 h at pH 7–9

Each hydrolysate solution was brought to 90°C for 15 min immediately after the hydrolysis process to inactivate the enzymes. The muscle hydrolysates and intact protein were stored at −80 °C and later freeze-dried for subsequent analyses. Both muscle hydrolysates and intact muscle were sent to the Ohio State University for proteomic analyses.

Diets

Five diets were formulated to contain varying mixtures of different hydrolysates with intact protein. The diets were formulated to be isonitrogenous and isolipidic and meet the essential nutrient requirements of larval fish (NRC, 2011). The diet formulations are presented in Table 2.1. Prior to mixing, the dry components of the diet were ground to a fine particle size (~0.25 mm) using a centrifugal mill (Retsch 2 M 100, Haan, Germany). Once the dry components were ground, all ingredients in the diet were mixed (HCM450 Vertical Cutter Mixer, Hobart, Troy, OH, USA) to achieve uniform dispersion. The mixture was extruded (Caleva Extruder 20, Sturminster Newton Dorset, England) to produce “noodles” and the

noodles were freeze-dried (Labconco, Kansas City, MO, USA) to remove moisture from the diets. After drying, all pellets were separated by size using a vibratory sieve shaker (Retsch AS 200 Basic, Haan, Germany).

The first diet, the intact diet, did not contain any of the LMB muscle hydrolysates and was solely based on LMB intact protein. The dietary protein in the last four diets was based on 50% intact protein and 50% LMB muscle hydrolysates (Kwasek et al. 2021). Diet A contained an equal proportion of all three of the hydrolysates. Diet B contained a 1:3:6 proportion of the 1.5, 3, and 6 h hydrolysates, respectively. This diet was formulated to have the highest proportion of the most hydrolyzed muscle; thus, it contained higher levels of lower-molecular-weight protein products than the other diets. Diet C contained a 1:3:1 proportion of the 1.5, 3, and 6 h hydrolysates, respectively. This diet had a higher proportion of medium-level hydrolysate protein and, therefore, fewer smaller protein fragments than diet B. The last diet, diet D, contained a 6:3:1 proportion of the 1.5, 3, and 6 h hydrolysates, respectively. This diet had a higher proportion of the least hydrolyzed muscle and more of the larger protein fragments than the other hydrolysate-based diets. The dietary amino acid compositions are presented in Table

Experimental Design

At 4 days post-hatch (dph), larval LMB (Little Grassy Fish Hatchery, Makanda, IL, USA) were randomly distributed into 21 (280 L) tanks, with ~10 fish/L. There were seven treatment groups in this study, with three replicate tanks each. Each group was fed to apparent satiation. The feeding regimen for each group is laid out in Figure 2.1. The experiment was carried out until the fish fully metamorphosed into the juvenile stage (5–26 dph). For the groups with changing diets throughout the study, the diets were switched after weighing the fish at the end of each week. The diets were ground up and fed to the fish in powder form (<250 µm). To

ensure constant feed availability during the first week, the larvae were fed every 45 min. After that, during the second week, fish were fed every hour, and during the third week, every hour and a half. Due to high viscosity, the dry feed was added to the surface of the tanks through a sieve (250 µm) to avoid the formation of clumps and ensure proper particle size for consumption. Feed was added to the tank in excess during each feeding. For the tanks that received live feed, *Artemia* was added to the tanks ad libitum and was monitored hourly to ensure a constant supply of food throughout the day.

The **Hydro-A** group received diet A throughout its development. The next two groups received a series of diets B, C, and D. This is due to the results provided by Canada et al. (2017), who found that as larvae develop, their ability to digest and absorb different protein sizes changes. Each of the different hydrolysate-based diets contained varying levels of hydrolyzed protein and, thus, varying molecular sizes of protein. **Hydro-BCD** received a series of hydrolysate-based diets throughout larval metamorphosis that started with diet B during week 1, switched to diet C for week 2, and finished with diet D during week 3. This dietary regimen was applied in order to determine whether providing larval fish with protein in a smaller-molecular-weight form and shifting toward larger sizes further into development would improve digestion and utilization of the proteins from the feeds. The next group, **Hydro-DCB**, was the opposite of the previous group; here, we tested how fish would perform when receiving larger protein fractions at first and then smaller protein fractions as their development continued. These groups received dry diets only, without live food supplementation. The **Artemia** group served as a reference and provided a benchmark for larval growth and performance on only live food (**LF**). The final two groups, **LF-Intact** and **LF-Hydro BCD** were meant to represent how co-feeding with live food impacts the dietary utilization of intact and hydrolysate-based diets, respectively.

The **Intact** group received a diet based solely on intact protein, serving as a control for performance without the inclusion of LMB muscle hydrolysates.

Sampling and Measuring

The fish in this study were weighed weekly at the end of weeks 1, 2, and 3. In addition to obtaining the weight data, pictures of the larval LMB were taken during each weighing to gather weekly total length data. ImageJ software (Version 1.53t, NIH, Bethesda, MD, USA) was used to assess the length of fish from the pictures. At the conclusion of the study, 100 fish from each tank were assessed for lordosis, scoliosis, tail, and head/jaw deformities. These deformities are commonly seen in larval fish and are often used as an indicator of nutritional deficiencies (Cahu et al. 2003).

Samples for additional analysis were also taken at the conclusion of the study. Three fish from each tank were euthanized with an overdose of MS-222 and stored in RNAlater (#AM7021, Invitrogen by Fisher Scientific, Waltham, WA, USA) for the analysis of PepT1 expression in the gut. Samples for PepT1 gene expression were taken 24 and 2 h after feeding. Additionally, two sets of whole-body samples were taken from each tank, with three fish per sample. The sampled fish were euthanized in liquid nitrogen and stored at −80 °C for further FAA analysis. These sets of samples were taken 24 and 2 h after feeding and represented basal and postprandial FAA levels, respectively.

Free Amino Acid Analysis

FAA analysis of fish tissues was performed according to Kwasek et al. (2021). Muscle samples of three fish from each tank were combined and homogenized together with 0.1 mol/L HCl in 1:9 (w/v) and spun at 12,000× *g* (4 °C, 15 min). Supernatants were collected, filtered (Milipore, 10 kDa cutoff at 15,000× *g*, 4 °C, 30 min), and later diluted with 0.1 mol/L HCl (1:19

v/v) containing norvaline and sarcosine (40 μmol/L) as internal standards. Blanks (0.1 mol/L HCl + 40 μmol/L norvaline and sarcosine) and external standards (Sigma acid/neutral and basic AA) were prepared along with the sample preparation. The same concentration of glutamine in 0.1 mol/L HCl as an external standard was prepared and added to the basic AA standard. Free amino acids were quantified using Shimadzu Prominence Nexera—i LC-2040C Plus (Shimadzu, Japan) according to the Shimadzu protocol No. L529 with modifications. Free amino acid concentrations (expressed as μmol/kg wet body weight) were calculated in LabSolutions software version 5.92 (Shimadzu, Japan) using internal and external standards.

Gene Expression

Gene expression was analyzed using real-time polymerase chain reaction (RT-PCR) as described in Terova et al. (2009). Before the transcript quantification of the PepT1 gene in the LMB intestine, PepT1 in LMB was molecularly cloned and sequenced as the sequence was not available in the GenBank database.

The primers for the amplification of a partial cDNA sequence of the PepT1 gene in LMB were designed based on the sequence of European sea bass (*Dicentrarchus labrax*) (GenBank acc. n° FJ237043.2). The sequences of the primers (351 bp) were: 5′-GATGACTGTGGGGATGTTCC -3′ for the forward primer and 5′-TCCGGCTTTGATTTGATGTCT -3′ for the reverse primer.

For PCR amplification, an aliquot of cDNA from the LMB intestine was amplified using Advantage® 2 Polymerase Mix (#639201, Takara Bio USA, Inc.) and the designed primers. Thirty-five PCR amplification cycles were set using the T100 Thermal Cycler (BioRad, Segrate, Italy). For cloning, PCR thermal cycling conditions were: initial denaturation at 95 °C for 1 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min, and a final elongation

step at 69 °C for 1 min followed by 10 min at 70 °C as suggested for T/A cloning. The PCR amplification product was then loaded onto 2% agarose gel with ethidium bromide in TAE (Tris, Acetic Acid, EDTA) 1X buffer to verify the amplicon length. The band of interest was extracted from the gel and purified using the NucleoSpin® Gel, and PCR Clean-up kit (Macherey-Nagel, Germany). Then, the DNA (insert of interest) was directly ligated to the T-tailed plasmid vector pGEM®-T Easy (Promega Milan, Italy) and subsequently sequenced in both directions (T7 and SP6). The alignment with Clustal Omega software of partial PepT1 cDNA sequence from LMB with PepT1 from *D. labrax* showed a high degree of similarity (78%) between the two fish species. Hence, the primers designed on the *D. labrax* sequence were found to be suitable for amplifying the PepT1 gene in LMB cDNA samples, too.

The RT-qPCR was carried out in a final mix volume of 20 µL, containing 3 µL of LMB cDNA (100 ng), 10 µL of iTaq Universal SYBR® Green Supermix (#1725121, Bio-Rad, Milan, Italy), and 500 nM of each primer, using the CFX96 RT-PCR instrument (Bio-Rad, Milan, Italy), following the manufacturer's instructions. The reaction thermal conditions were as follows: 95 °C for 1 min, then 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. A blank sample containing nuclease-free water instead of the cDNA template was included in each assay as a negative control. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and *eEF1a1* as housekeeping (HK) gene. With regard to HK, we tested three constitutive genes, α-tubulin, *eEF1a1*, and *β-actin*, to select the most stable one to normalize the relative quantification of PepT1 expression. Primers for the tested HK genes were designed based on the sequences of each gene available in the Genbank database for LMB, whereas primers for the amplification of the LMB PepT1 gene were designed based on the aforementioned partial cDNA sequence obtained by molecular cloning and sequencing.

The accession numbers of the sequences used to design primers for HK genes were as follows: XM_038724778.1 for *eEF1a1*, MH018566.1 for *α-tubulin*, and MH018565.1 for *β-actin*. Primers were designed following the instructions given in the SYBR Green Supermix kit. The melting temperature was 60 °C. The open-source Primer3 under default settings was used to design the primers. Moreover, for the best qPCR efficiency, amplicon length for each pair of primers was set between 70 and 150 bp. Primers used to amplify target and HK genes are listed in Table 2.3.

By applying the CFX Maestro™ Software (Bio-rad, Milan, Italy), the best reference gene was selected and its stability analyzed by means of the reference gene selection tool (CFX Maestro™ Software User Guide Version 1.1, Bio-rad, Milan, Italy). Following this analysis, the software indicated that *eEF1a1* was the ideal HK based on the average M value (M = 0.48) (Figure 2.2).

Amplification reaction efficiency is another important parameter to be considered when performing relative quantitation. Therefore, it was tested for each primer set using, again, CFX Maestro™ Software (Bio-rad, Milan, Italy). Amplification efficiency of a reaction is calculated according to the following equation using data collected from a standard curve: Exponential amplification = 10 (−1/slope)

For this, serial dilutions of LMB cDNA were prepared and a standard curve was constructed for each pair of primers. Typically, desired amplification efficiencies range from 90% to 110%. In our case, each primer set showed an amplification efficiency > 80% (Figure 2.3).

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to visualize the products of muscle hydrolysis. Electrophoretic analysis of the samples was conducted on a discontinuous, reducing 12.5% T, SDS-PAGE with modifications of the method described by Updike et al. (2006). Samples were homogenized in dissociation buffer (8 M urea, 2 M thiourea, 60 mM Tris, pH 6.8, containing 3% SDS, 350 mM DTT, and 0.002% bromophenol blue) overnight at 25 °C with agitation and diluted with dissociation buffer as needed. Ten µL of the sample (10 mg/mL) were loaded onto each lane with a 3% stacking gel containing 1% SDS. The proteins were resolved at 150 V cm^{-1} until the dye front reached within 1 mm of the bottom of the gel. The gel was stained with Coomassie Brilliant Blue dye (40% methanol, 5% acetic acid, 0.04% Coomassie Brilliant Blue G-250) overnight and destained with 10% acetic acid. After destaining the gel image was digitized using an AZURE c600 scanner (Azure Biosystems, Dublin, CA, USA).

Statistical Analysis

The growth performance, survival, and skeletal deformity data collected from this study were analyzed using one-way ANOVA and a Tukey test was used to detect differences between groups. The FAA and PepT1 data were analyzed using a one-way ANOVA and differences between groups were tested using an LSD test. Gene expression variations were analyzed by ANOVA using the PAST3 program (Palaeontological Statistics). In the event of significant differences between the means, the post hoc Tukey or Dunn's test was applied. The level of significance was set at $p < 0.05$.

RESULTS

LMB Muscle Hydrolysates

Figure 2.4 shows the results of the LMB muscle hydrolysates obtained using endogenous digestives enzymes from adult LMB on a 12.5% SDS-PAGE gel. This concept of hydrolysis has been demonstrated prior by Kwasek et al. (2021). This electrophoretic analysis was performed to confirm the products used in this study were hydrolyzed and to observe any differences that the incubation time had on the hydrolysates. The results from this gel show that the starting material was intact prior to digestion (Lane 2). This demonstrates that without incubation, very little hydrolysis occurred in the time period of harvesting, mixing with the enzymes, and increasing the temperature to 95 °C (heat shock). The intact LMB muscle was incubated with enzymes and samples removed at 1.5 h (Lane 3), 3 h (Lane 4), and 6 h (Lane 5). The hydrolysates were analyzed to observe the degree of hydrolysis over time. Based on these data, the supernatant shows a presence of proteins smaller than 65 kDa initially at 1.5 h. Peptides less than ~45 kDa were remaining after incubation of 3 h and by 6 h it appears that hydrolysis was complete with no observable peptides remaining. This suggests that the proteins had been reduced to small peptides of lower than ~25 kDa. Additionally, this gel confirms that the in vitro muscle hydrolysis process was able to hydrolyze the LMB muscle used in this study.

Growth and Survival

After the first week of the study (12 dph), the Artemia group had a significantly higher average total length than all other groups. The two LF groups had a significantly higher average total length than the four dry feed-only groups. Among the dry feed-only groups, the Hydro-BCD group had a significantly higher average total length than the other groups, and the Intact group had a significantly higher average total length than the Hydro-DCB group. After the

second week (19 dph), the average total lengths of the Artemia, LF-Hydro, and LF-Intact groups were significantly higher than all other groups. Additionally, the Intact group had a significantly higher average total length than the Hydro-BCD and Hydro-DCB groups. At the conclusion of the study (26 dph), the average total length of the LF-Intact group was significantly higher than all other groups, and the LF-Hydro and Artemia groups had significantly higher average total lengths than the four dry feed-only groups. Within the dry feed-only groups, the Intact group had a significantly higher average total length than the Hydro-DCB group. The average total lengths of the Hydro-A and Hydro-BCD groups were not significantly different from either the Intact or Hydro-DCB groups. The average total length results for each week are presented in Figure 2.5.

At the conclusion of the study (26 dph), the LF-Intact group had a significantly higher average weight than the Hydro-A, Hydro BCD, and Hydro-DCB groups. However, there were no significant differences in final weight among the LF-Hydro, LF-Intact, and Artemia groups. The Artemia group had a significantly higher survival than all other groups, while the LF-Intact group had a significantly higher survival than the LF-Hydro group and the four dry feed-only groups. The LF-Hydro group had numerically higher survival than the Intact group (~10% increase); however, the difference was not significant. The LF-Hydro and Intact groups had significantly higher survivals than the three hydrolysate-based dry feed-only groups. The results for growth performance and survival are presented in Table 2.4.

Skeletal Deformities

The occurrence of total skeletal deformities (scoliosis, lordosis, head/jaw, and tail) in the intact group was significantly higher than in all other groups, while there were significantly more deformities in the LF-Intact and Hydro-A groups than in the remaining groups. The LF-Hydro group had a significantly lower occurrence of skeletal deformities compared to all other groups

except the Artemia group. There was no significant difference in skeletal deformities between the Hydro-BCD and Hydro-DCB groups. The results for skeletal deformities are presented in Figure 2.6.

PepT1 Expression

There were no significant differences in PepT1 gene expression between any of the groups 24 h after feeding. However, gene expression of PepT1 was significantly higher in the Hydro-DCB group compared to every group except Hydro-A and Hydro-BCD 2 h after feeding. The results for PepT1 expression are presented in Figure 2.7.

Free Amino Acid Composition

The LF-Hydro group presented with significantly higher postprandial levels of total FAA and IDAA in the muscle than the Artemia and LF-Intact groups (Figure 2.8). The Hydro-DCB group had significantly higher levels of total DAA than the Artemia, Intact, and Hydro-A groups. All groups that received dry feed, except Hydro-A, had a significantly higher level of free lysine than the Artemia group. Significantly higher levels of free threonine were measured in the muscle of the LF-Hydro group than in all other groups, and all groups that received dry feed, except Hydro-A, had a significantly higher level of free threonine than the Artemia group. The Hydro-A group had higher levels of free methionine than the LF-Intact group but did not differ significantly from those in the remaining groups. The Hydro-DCB group showed significantly higher levels of free aspartic acid compared to the Artemia group, and a significantly higher level of free asparagine than the LF-Intact group. The LF-Hydro and LF-Intact groups had significantly higher levels of free glutamic acid compared to the Hydro-A group, while the LF-Hydro group also showed levels significantly higher than those in the Artemia group. The Hydro-BCD and Hydro-DCB had significantly higher levels of free serine than all other groups,

except the LF-Hydro group. The Intact group had a significantly higher level of free glutamine than the Hydro-A group. The Artemia group had a significantly lower level of free histidine, tyrosine, arginine, and phenylalanine than all other groups. The LF-Hydro group had a significantly higher level of free glycine than all groups, except the LF-Intact group. The LF-Hydro group also had significantly higher levels of both free arginine and tyrosine than all other groups, except the Hydro-BCD group. The postprandial level of free alanine in the muscle was highest in the Hydro-DCB group, significantly higher than all groups except LF-Hydro and Hydro-BCD. The Hydro-BCD group had the highest level of free phenylalanine, significantly higher than all other groups except for the Intact group. The Hydro-BCD group also had the highest level of free tryptophan, significantly higher than the LF-Hydro, Hydro-A, and Artemia groups. The postprandial level of free proline in the muscle was highest in the Hydro-DCB group, significantly higher than all other groups except for Hydro-A. No significant differences were observed in the postprandial levels of free leucine, isoleucine, valine, and cysteine between groups. The postprandial results for all FAA analyzed are presented in Table 2.5 (2 h) and Table 2.6 (24 h).

DISCUSSION

Kwasek et al. (2021) developed an in vitro hydrolysis method utilizing endogenous digestive enzymes from adult LMB. The study found that dietary inclusion of Bighead carp muscle hydrolyzed with the LMB endogenous enzymes significantly improved the growth of larval LMB at a 37% inclusion level (Kwasek et al. 2021). The present study utilized the same in vitro hydrolysis method using LMB endogenous digestive enzymes, and instead of hydrolyzing carp muscle, hydrolyzed the muscle from adult LMB to provide the larval LMB with an optimal dietary amino acid profile. Additionally, the present study utilized LMB muscle that underwent

varying degrees of hydrolysis, to provide a wider range of molecular weight of protein products in the diets that would account for the dynamics associated with larval LMB digestive tract development. In contrast to Kwasek et al. (2021), the results of the present study did not show a significant improvement in overall growth or survival by including the hydrolysate. The survival results observed in this study are comparable to other studies that utilized protein hydrolysates. Sheng et al. (2022) obtained survival levels between 29–43% in larval LMB that were co-fed with live feed during the first five days of the trial, compared to the 33.78% and 46.29% survival levels observed in the co-fed groups (LF-Hydro and LF-Intact) in the present trial. The survival levels observed in the four groups fed dry diets only were markedly lower, ranging from 9–24%. While this survival is very low, it provides an improvement over previous studies that fully replaced live feed with formulated dry diets in Senegalese Sole (Canavate and Fernández-Diaz, 1999) and Red Seabream (*Pagrus major*) (Khoa et al. 2020), both of which experienced no survival within 15 days of feeding with dry diets only. Interestingly, the survival in the hydrolysate-based groups was significantly lower than in the corresponding intact-based groups (i.e., LF-Intact vs. LF-Hydro, and Intact compared to the three hydrolysate-based dry feed groups). This reduction in survival could be attributed to physical differences between the feeds. One major hindrance to weaning larvae onto dry feed is the shortened availability of feed pellets in the water column and lack of predatory response trigger, reducing feed intake (Rasdi and Qin, 2016; Samat et al. 2020). Although all the diets were processed in the exact same way, it was observed that the intact diet seemed to disperse more evenly at the surface and sink slower through the water column than the hydrolysate-based diets. This difference in the behavior between the feeds may have increased the feed intake for the groups fed with the intact diet, which could have contributed to the increased survival in the intact groups as compared to the

hydro groups. Additionally, the solubility of proteins has been found to rise with increasing degrees of hydrolysis, leading to increased nutrient leaching in the water (Carvahlo et al. 2004; Önal and Langdon, 2009; Martínez-Alvarez et al. 2015). This leaching lessens the nutritional value of the diet and reduces the levels of dietary protein available to the larval fish (Martínez-Alvarez et al. 2015). Although nutrient leaching from diets to some extent was expected and was accounted for through smaller and more frequent feedings, it is possible that more leaching occurred from the hydrolysate-based feeds, compromising the amino acid composition of the diet and inducing higher mortality in the groups fed with those diets due to potential nutritional deficiency.

Another possible and more convincing explanation for the difference in results is that Kwasek et al. (2021) used only a 3 h hydrolysate in their diets, while this study used a mix of 1.5-, 3-, and 6 h hydrolysates. The composition of protein fragments has a significant impact on the growth of larval fish (Kotzamanis et al. 2007; de Vareilles et al. 2012; Srichanun et al. 2014). De Vareilles et al. (2012) found that a 15% inclusion of larger polypeptides along with 5% of smaller protein sizes (di-/tripeptides, FAA) increased the growth of larval White Seabream (*Diplodus sargus*), while 15% inclusion of the small protein fragments and 5% of the large peptides reduced the growth of the larval Seabream. The mix of hydrolysates in this study may have been over-hydrolyzed and provided levels of small peptides and FAAs in the diet that were too high, ultimately reducing protein absorption and utilization. This reduction in dietary protein uptake has been attributed to two major factors, an oversaturation of the intestinal protein transporters (Rønnestad et al. 2000; Zhang et al. 2006; Ambardekar et al. 2009) and the asynchronous absorption of dietary amino acids (Rønnestad et al. 2000; Ambardekar et al. 2009). The former increases the excretion of amino acids due to their inefficient uptake and the latter

reduces protein synthesis due to an imbalanced FAA pool (Rønnestad et al. 2000; Carvahlo et al. 2004). Additionally, Kwasek et al. (2021) utilized Bighead Carp muscle as a protein source instead of LMB muscle, which could have also led to differing results. It has been shown that the protein source of hydrolysates used in larval diets significantly affects survival rate (Srichanun et al. 2014). These differences highlight how important it is to determine not only the optimal muscle source, but also the optimal composition of proteins with varying molecular weights when formulating diets for larval fish. While intact protein has been found to be more difficult to digest and utilize by larval fish, compared to hydrolyzed protein, there is a point where a hydrolysate can be over-hydrolyzed and becomes less efficient than the intact protein.

Although we did not observe positive results on overall growth performance and survival in our study, a significant reduction in skeletal deformities occurred with the inclusion of the protein hydrolysate. These deformities, such as scoliosis and lordosis, have been associated with vitamin (Roy and Lall, 2007; Fernández et al. 2008), phospholipid (Kanazawa et al. 1982), and amino acid deficiencies (Akiyama et al. 1986a). A high rate of skeletal deformities has a negative impact on production levels at hatcheries, as the deformed fish experience significantly stunted growth and, in more extreme cases, the fish die (Boursiaki et al. 2019). In a strictly industrial sense, successful larval rearing would be defined as producing larger and healthier juveniles and adults that can be sold for human consumption or stocking. Fish that have significant deformities are typically disposed of or sold at significantly reduced prices, leading to economic losses for the farmers (Fernández et al. 2008). The reduction in deformities observed in this study is in agreement with previous studies that investigated hydrolysate inclusion in the diets of larval fish (Zambonino Infante et al. 1997; Cahu et al. 1999; Johannsdottir et al. 2014; Peruzzi et al. 2018; Kwasek et al. 2021). Cahu et al. (1999) found that 11% fewer skeletal

deformities developed in sea bass larvae with a 58% inclusion of a fish protein hydrolysate in the feed, compared to a diet based solely on intact protein. As observed in this study, the reduction in skeletal deformities does not always result in improved growth performance and survival. Although the LF-Hydro group had a significantly lower level of skeletal deformities, it also had significantly lower weight and survival at the conclusion of the study, compared to the LF-Intact group. Additionally, within the four dry feed-only groups, the Intact group had a significantly higher occurrence of skeletal deformities than the three groups that received the hydrolysate-based diets but had twice the survival. Similarly, Cahu et al. (1999) found that the occurrence of skeletal deformities was reduced in larval Sea Bass with increasing inclusion levels of hydrolysates from 19% to 38% to 58%. However, the inclusion levels of 38% and 58% resulted in significant reductions in survival and growth in the Sea Bass (Cahu et al. 1999). This would signify that there seems to be a trade-off between promoting positive growth response and survival versus supporting optimal skeletal development when it comes to the right inclusion level of protein hydrolysates. For example, the LF-Intact group produced the longest and heaviest fish, with 46.29% survival, but 44.23% of the surviving fish exhibited skeletal deformities. Compare that group to the LF-Hydro which experienced a ~12% reduction in weight, 13% lower survival, but a ~33% decrease in skeletal deformities.

One previously mentioned cause of the reduced growth associated with high levels of low molecular weight protein in the diet is a rapid and asynchronous absorption of dietary amino acids into the body (Rønnestad et al. 2000; Ambardekar et al. 2009). The absorption of dietary amino acids was analyzed in this study by measuring the postprandial FAA composition in the muscle. Previous studies have utilized this parameter as a measure of the availability of dietary amino acids (Kwasek et al. 2009), the quality of dietary protein (Kaushik and Luquet, 1980;

Mente et al. 2003), and the impact of the molecular form of dietary protein (Zhang et al. 2006). In this study, an interesting result was the increase in total FAA and IDAA in the muscle 2 h after feeding in the LF-Hydro group, compared to the LF-Intact and Artemia groups. The postprandial increase in FAA in the LF-Hydro group would seem to signify a higher level of absorption of the dietary amino acids from the hydrolysate-based diet compared to the intact-based diet. However, this increase in TFAA and IDAA did not result in an increase in growth compared to those two groups, and thus did not result in increased protein synthesis. This could be due to the asynchronous absorption of the dietary AA, specifically among the IDAA. According to Kwasek et al. (2021), the appearance of FAA in the muscle of larval LMB fed with a hydrolysate-based diet seemed to peak earlier than the 3 h postprandial sampling time, compared to the LMB fed with an intact-based diet. Additionally, the authors reported that the FAA in the group fed the intact-based diet peaked around the 3 h sampling time, which would be after the 2 h postprandial sampling time in this study. This is supported by the findings in this study, which show significantly higher levels of TFAA and IDAA at 2 h after feeding in the LF-Hydro group, compared to the LF-Intact group. Since it can be assumed that the muscle FAA has peaked in the LF-Hydro group, one possible explanation for the observed decrease in growth is a delayed peak in threonine compared to the other IDAA. At 2 h after feeding, the threonine level in the LF-Hydro group is only slightly higher than the basal level, while the other IDAA have shown substantial postprandial increases, compared to the basal level. Threonine is one of the three major limiting amino acids in fish, meaning amino acids within the muscle can only be utilized for protein synthesis up to the available level of threonine. This possibly suggests that although the LF-Hydro group had significantly higher postprandial levels of TFAA and total

IDAA, the delayed availability of threonine might have been one of the factors that limited protein synthesis and increased protein catabolism.

Part of the novel approach of this study was also utilizing a series of diets that presented the larval LMB with increasing dietary protein sizes throughout development. This was based on Canada et al. (2017) who found that dietary protein size requirements in larval Senegalese Sole shift from partially hydrolyzed protein (5–70 kDa) before and during metamorphosis to intact protein after metamorphosis. This was reflected by significantly improved growth in the Senegalese Sole, during the metamorphic stage, fed with a diet based on the partially hydrolyzed protein, compared to those fed with the intact protein (Canada et al. 2017). After the metamorphic stage, the diet based on intact protein significantly improved the growth of the fish (Canada et al. 2017). Based on this outcome, it was suggested that dietary protein should be optimally transitioned from smaller to larger protein fractions over the course of development during the larval stage. This theory was tested in the present study with the Hydro-BCD group, which received a series of diets formulated with increasing protein sizes throughout the feeding trial. The overall results from this group showed that the series of diets provided did not improve the growth or survival of the larval LMB. However, the weekly growth results obtained from this study do support the findings in Canada et al. (2017). After the first week of feeding, the Hydro-BCD group had a significantly higher average total length than the other dry feed-only groups. This suggests that Diet B, which was formulated to have the highest composition of lower molecular weight proteins, was the most efficient diet during the first week of feeding for larval LMB. After the second week of feeding, however, the Hydro-BCD group had a significantly lower average total length than the Intact group. The significant increase in growth of the Intact group during the second week might suggest that at this point in the development of larval LMB,

the fish were better suited to feed on diets with larger protein fragments, and the molecular weight profile of Diet C was too low to support optimal growth for the Hydro-BCD group. Previous studies have found that the inclusion of hydrolysates in larval diets stimulated the maturation of the gut and the activity of enzymes associated with protein digestion (Cahu et al. 1999; Kotzamanis et al. 2007). This maturation of the gut is characterized by a reduction in peptidases and an increase in the activity of enzymes, such as pepsin and trypsin, that break down intact proteins into smaller protein fragments for absorption (Dabrowski, 1984; Kolkovski, 2021). Based on this, it seems that the initial use of hydrolysates does improve the growth and development of larval LMB; however, after a certain point in the gut maturation, the continued use of hydrolysates becomes inefficient for protein utilization. This is supported by the significant difference in the utilization of Diet B at different points of larval development. Diet B significantly increased the growth performance of the larval LMB during the first week (Hydro-BCD vs. Intact); however, the use of Diet B during the final week of the study significantly decreased the growth of the LMB (Hydro-DCB vs. Intact) and was less efficient than the intact diet. This highlights the importance of providing larger dietary proteins as the LMB progress through larval development.

While we observed that LMB did utilize increasing protein fragments more efficiently throughout development, the series of diets that were intended to provide the LMB with increasing molecular weight proteins were unable to improve the growth performance over the group that received solely the intact diet. As previously mentioned, we assume that the hydrolysates utilized in this study were over-hydrolyzed and provided dietary protein molecular weight profiles that were much smaller than intended. Canada et al. (2017) found that their diet with a high level of low-molecular-weight peptides (<5 kDa) significantly reduced the growth of

the larval Senegalese Sole compared to the group that received the partially hydrolyzed protein. Previous studies suggest that high inclusion levels of small peptides and FAA lead to an oversaturation of intestinal transporters, reduced protein absorption, and, ultimately, reduced growth (Kotzamanis et al. 2007; Canada et al. 2017). It is feasible that both the 3 and 6 h hydrolysates were over-hydrolyzed and provided the diets with too high of a level of low-molecular-weight proteins. This would suggest that diets with high inclusion levels of those two hydrolysates (Diets A, B, and C), although formulated to be different, had molecular weight profiles that were lower than intended and utilized similarly for growth. This theory helps explain the lack of significant differences observed in growth and survival between the Hydro-BCD, Hydro-DCB, and Hydro-A groups. One result observed that signifies the similarities in molecular weight profiles of Diet A and Diet B might be the increase in postprandial expression of PepT1. PepT1 results from Diet C were not obtained as it was not being fed to any of the groups at the conclusion of the study when PepT1 samples were collected. The Hydro-DCB group had a significantly higher expression of PepT1 2 h after feeding compared to all other groups except the Hydro-A and Hydro-BCD groups. Additionally, the Hydro-A group had a numerically higher postprandial expression of PepT1 compared to all other groups, except Hydro-DCB. These results might indicate an increase in di-/tripeptide absorption from diets A and B, the two diets with the highest inclusion levels of the 3 and 6 h hydrolysates. However, the increase in peptide absorption was not reflected in improved growth performance in this study.

Overall, the results from this study show that the inclusion of the same-species muscle hydrolysate improved the initial growth of larval LMB but did not improve the overall growth throughout the entire larval stage. It seems that the hydrolysates utilized in this study were over-hydrolyzed and were unable to support optimal growth and survival in larval LMB throughout

the fish metamorphosis. To improve the use of same-species muscle hydrolysates as a protein source for larval fish, further research should look into optimizing the hydrolysis process to produce a more ideal molecular weight profile and improve the physical qualities of the diet to ensure high nutrient retention.

CHAPTER 3

DOES THE SOURCE OF MUSCLE AND/OR DIGESTIVE ENZYMES AFFECT THE QUALITY OF FISH PROTEIN HYDROLYSATES USED AS FIRST FEED FOR LARVAL FISH?

ABSTRACT

Fish protein hydrolysates have been used in the past as a source of protein in larval fish diets with inconsistent success. The protein hydrolysates used in the larval diets have been produced from a wide variety of species and used various enzymes to hydrolyze the protein. The objective of this study was to compare the effect of dietary inclusion of a fish muscle hydrolysate produced from species-specific muscle and enzymes to hydrolysates produced from those of a different species. The quality of each hydrolysate as a dietary protein source was assessed based on its effect on; 1) growth performance and survival; 2) the occurrence of skeletal deformities; 3) expression of intestinal peptide transporter PepT1; and 4) the muscle free amino acid (FAA) pool; in larval Walleye (*Sander vitreus*). Four hydrolysates were produced for this study, two obtained from Walleye muscle, one hydrolyzed with endogenous enzymes obtained from Walleye and another one with Nile Tilapia (*Oreochromis niloticus*) endogenous enzymes. The other two hydrolysates were obtained from Tilapia muscle, hydrolyzed with either the Walleye or Tilapia endogenous enzymes. The muscle and enzymes were mixed continuously for 3 h during the hydrolysis (at 22^{o}C and 28^{o}C for Walleye and Tilapia enzymes, respectively), and the pH was adjusted throughout the process to mimic gastric and intestinal digestion conditions. Additionally, an intact protein source was produced using each muscle/enzyme combination. Four diets were produced with the dietary protein supplied as a 50/50 ratio of the intact and hydrolyzed muscle from the respective muscle/enzyme combination. At 5 days post hatch (dph),

Walleye larvae were randomly distributed to 18 (280 L) tanks containing ~9 fish/L. In this study, there were six treatment groups, each with three replicate tanks. Four groups were fed one of the formulated diets produced in this study, and two additional groups of larvae fed a commercial starter diet and *Artemia*, respectively, were included as reference groups. The larvae were fed in excess, 9 times a day. The experiment was conducted until the fish fully metamorphosed into the juvenile stage (26 dph). The larval Walleye that received the diet with muscle hydrolysate produced with Walleye muscle and Walleye endogenous enzymes had a significantly higher average weight at the conclusion of the study than the other groups. Additionally, a significant interaction effect was observed between muscle and enzyme source on the growth of larval Walleye. The species-specific combination also led to a significant increase in the postprandial total FAA and IDAA content in muscle FAA pool. There were no significant differences observed between the hydrolysate-fed groups in the survival rate, occurrence of skeletal deformities, or the expression of intestinal peptide transporter PepT1. Each hydrolysate-based diet led to a significant reduction in skeletal deformities and survival, compared to a group fed with a commercial diet. Overall, the results from this study suggest that species-specific muscle and enzymes produce a more optimal dietary protein source for larval fish than non-species-specific products, but further research should focus on improving the physical properties of the formulated diets to reduce possible leaching of the hydrolyzed protein and improve survival of fish larvae.

INTRODUCTION

To improve utilization of dietary protein in fish larvae with underdeveloped digestive tracts, protein hydrolysates have been added to formulated larval diets as a substitute for intact protein. Hydrolysates from purified protein sources, such as casein (Szlaminska et al. 1991; Cahu

and Infante, 1995; Carvalho et al. 1997) and collagen (Liu et al. 2019), and those from more practical ingredients, such as fish (Tonheim et al. 2005; Kotzamanis et al. 2007; Kvåle et al. 2009) and plant meals (Srichanun et al. 2014) were investigated. Among the hydrolysates tested, fish protein hydrolysates appear to be the most suitable for fish larvae particularly due to their complete amino acid profile and palatability enhancing properties (Kasumying and Doving, 2003; Refstie et al. 2004). Previous studies have compared the use of fish protein hydrolysates with hydrolysates from other animal sources, such as shellfish and invertebrates (Srichanun et al. 2014; Bui et al. 2014). Although fish protein hydrolysates have been shown to be the most efficient (Srichanun et al. 2014), research comparing the use of protein hydrolysates from different species is limited. Given the significant differences in proximate composition, amino acid profile, and overall quality of muscle protein between fish species (Pyz-Łukasik and Paszkiewicz, 2018; Ryu et al. 2021), the species source of protein appears to be critical for producing an ideal protein hydrolysate for larval fish.

Chemical hydrolysis and enzymatic hydrolysis are the two main methods used to produce hydrolysates, with enzymatic hydrolysis being preferred to obtain a higher nutritional value of the protein hydrolysate, with more specific peptide-bond cleavages that produce more consistent amino acid profiles (Clemente, 2000; Zamora-Sillero et al. 2018; Sandbakken et al. 2023). Typically, protein hydrolysates are produced utilizing commercial enzymes, such as Protease N®, Alcalase®, and Neutrase®, or with enzymes extracted from plants, such as papain, bromelain, and ficin (Kristinsson and Rosco, 2000; Aspmo et al. 2005; Ryu et al. 2021). The selection of these enzymes is important because previous studies have shown that hydrolysis of the same muscle with different enzymes has significant effects on its antioxidant properties, nutritional quality, degree of hydrolysis, and amino acid composition (Hathwar et al. 2011; He et

al. 2013; Saidi et al. 2013; Elavarasan et al. 2014; Ryu et al. 2021). In addition to commercial enzymes, studies have investigated the use of endogenous enzymes from fish viscera to produce hydrolysates (Aspmo et al. 2005; Herpandi et al. 2011; Silva et al. 2014; Kwasek et al. 2021; Molinari et al. 2023). These endogenous enzymes are considered more efficient for the production of protein hydrolysates and would reduce the costs associated with the use of commercial enzymes (Kristinsson and Rasco, 2000).

The species source of endogenous enzymes is critical to producing an optimal hydrolysate, as the digestive physiology of each species differs significantly. Carnivores are characterized by larger stomachs, shorter intestines, and higher levels of proteases (Buddington et al. 1997). In contrast, the digestive tracts of omnivores/herbivores generally have longer intestines, with smaller (or absent) stomachs, and higher levels of carbohydrases (Kramer and Bryant, 1995; German and Horn, 2006). The use of species-matched endogenous enzymes appears to be the most effective hydrolysis method to obtain a high-quality hydrolysate for larval fish, as mimicking the *in vivo* digestion of proteins would compensate for their lower proteolytic activity. This method of using species-matched enzymes for *in vitro* hydrolysis has been shown to be successful (Kwasek et al. 2021).

In this experiment, we studied fish protein hydrolysates from the muscle of two different fish species, hydrolyzed with endogenous enzymes from these different species. We hypothesized that the combination of species-specific muscle and endogenous enzymes would yield a hydrolysate that had the ideal amino acid composition, in a form best suited for uptake and utilization by larval fish. The objective of this study was to determine the effect of muscle source and endogenous enzymes on the production of fish protein hydrolysates and their effects on 1) growth performance and survival, 2) the occurrence of skeletal deformities, 3) the

expression of the intestinal peptide transporter PepT1, and 4) the pool of free amino acids in (FAA) muscle, which serves as an indicator of dietary amino acid availability, in larval Walleye (*Sander vitreus*).

MATERIALS AND METHODS

Experimental Species

In this study, Walleye was used as an experimental fish. Walleye are considered cool-water carnivorous and stomach-possessing species that are a popular sport fish and are often raised as fingerlings for stocking. However, the production of Walleye as food fish is still limited in the United States. Most production occurs in ponds (Johnson et al. 2008). Pond culture of Walleye is problematic because it requires a lower optimal temperature for growth and has a very limited season for production (Johnson et al. 2008). One of the reasons that outdoor culture is still the preferred choice for Walleye production is that they rely on zooplankton as a food source when they first feed. Walleye are an altricial species, and although they have shown some acceptance of formulated feeds (Johnson et al. 2008), an optimal larval feed for this species has not yet been developed. Production of a formulated dry feed for Walleye larvae could help increase intensive Walleye production by reducing live feed and the need for outdoor ponds.

Nile Tilapia (*Oreochromis niloticus*) was used as a non-species-specific muscle and enzyme source because it is a species with distinctly different digestive and behavioral characteristics. Tilapia are tropical omnivores that have small stomachs (Morrison and Wright, 1999). In addition, Tilapia are a very popular aquaculture species, and their endogenous enzymes have been extracted and used in previous *in vitro* hydrolysis studies (Silva et al. 2014).

Experimental Conditions

The feeding trial was conducted at the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University-Carbondale (SIUC), IL. The experiment was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of SIUC. All protocols performed were approved by SIUC Institutional Animal Care and Use approved (protocol #22-019). The experiment was carried out using a semi-recirculated aquaculture system with two mechanical (sand) filters (Pentair, Minneapolis, MN) and one bio-filter. The system consisted of 30 black tanks (280 L). Photoperiod was maintained with overhead light strips and consisted of 24 h of dimmed light, with light intensity slightly increased during feedings. The system was supplied with groundwater, with a constant flow of 100 ml/min into each tank. During the study, the temperature was 20.38^{o}C (± 2.03) and the pH was 7.40 (± 0.55). The salinity of the system was maintained at 1-3 ppt to prolong the viability of the live feed (Dabrowski and Miller, 2018). Each tank was equipped with a sprinkler to break the water surface and allow Walleye larvae to inflate their swim bladders (Clayton and Summerfelt, 2010). Additionally, clay was added to the system each morning to increase the turbidity of the water. This was done to reduce cannibalism and clinging behavior in the tanks (Summerfelt and Johnson, 2015).

Muscle Hydrolysis

The hydrolysis method for this study was based on the *in vitro* method described in Kwasek et al. (2021), with some modifications. Eight different protein sources were produced, with one intact and one hydrolyzed protein obtained from the following combinations:

- Walleye muscle with Walleye digestive enzymes
- Walleye muscle with Tilapia digestive enzymes

- Tilapia muscle with Walleye digestive enzymes
- Tilapia muscle with Tilapia digestive enzymes

Both fresh and frozen muscles (50/50) and digestive tracts were used in this study. The fresh muscles and digestive tracts were harvested from adult Walleye and Tilapia that were kept at 22°C and 25°C, respectively. On the morning of harvest, the fish were fed to satiation and euthanized 2 h after feeding with an excess of tricaine methanesulfonate (MS-222) (#E10521, Sigma-Aldrich, St. Louis, MO, USA) at a dose of 0.4 mg/ml. The head and tail of each fish were severed, and the digestive tract was dissected out and placed on ice. The remaining carcass was minced three times with a meat grinder (General Food Service, Weston, FL, USA), diluted with deionized water (1:2), and homogenized with a PowerGen 1000 (Fisher Scientific, Waltham, MA, USA) tissue homogenizer at high speed for 10 min. The digestive tracts were also diluted with deionized water (1:2), homogenized for 10 min, and the homogenates were centrifuged (1500× *g* for 10 min at 4 °C) to recover the supernatant and to separate it from the solid mass (including undigested feed, fat, and tissues). The supernatant was then immediately used for muscle hydrolysis. The muscle homogenates were transferred to 12-liter containers, placed in a water bath at 25°C (for Tilapia enzymes) or 22°C (for Walleye enzymes), diluted with deionized water (1:2), and stirred with an overhead stirrer (VWR VOS 16, VWR, Radnor, PA, USA) for the duration of hydrolysis. These muscle homogenates were mixed with the digestive tract supernatant (3:1 muscle: supernatant). The hydrolyzed products were continuously stirred for 3 h and the pH was adjusted to mimic digestion. The pH was maintained at 3 to 4 for the first hour to mimic gastric digestion and then increased to 7 to 9 for 2 h to mimic intestinal digestion (Kwasek et al. 2021). After hydrolysis, the solution was brought to 90 °C for 15 min to stop enzymatic activity. The intact muscle products were brought to 90°C immediately after mixing to

prevent any hydrolysis. Although this product is not a fully intact protein, since it was mixed with endogenous enzymes for a short time, it is referred to as intact to distinguish it from the products that underwent the incubated hydrolysis process. All products were frozen at -20^{o}C and then freeze-dried to remove moisture. After freeze-drying, lipids were extracted from each muscle product by the Soxhlet method using ethyl ether as solvent (Ramluckan et al. 2014). Proteomic analysis of both intact and hydrolyzed products was performed at Ohio State University, Columbus, OH.

Diets

Four diets were formulated to be isonitrogenous and isolipidic and to meet the essential nutrient requirements of Walleye larvae or closely related species (NRC, 2011). They were formulated to contain 60% crude protein and 15% crude lipids. The formulations are listed in Table 3.1. The analyzed compositions of the feeds are shown in Table 3.2. The four feeds corresponded to the combination of muscles and enzymes used to produce the intact and hydrolyzed proteins:

- **W-W:** Walleye muscle with Walleye enzymes
- **W-T:** Walleye muscle with Tilapia enzymes
- **T-W:** Tilapia muscle with Walleye enzymes
- **T-T:** Tilapia muscle with Tilapia enzymes

The formulations for these experimental diets were based on a 50% replacement of intact protein with protein hydrolysates, similar to the formulations of Kwasek et al. (2021).

Experimental Design

At 5 days post hatch (dph), Walleye larvae were randomly distributed to 18 tanks (280 l) containing ~9 fish/L. In this study, there were six treatment groups, each with three replicate

tanks. The first four groups (**W-W**, **W-T**, **T-W**, and **T-T**) were fed one of the manufactured formulated diets and received the same-named diet for the duration of the feeding experiment. In addition, there was a live feed reference group that received only *Artemia* nauplii (**Artemia**) throughout the study, and a dry feed reference group that received a commercial starter feed (Otohime, Marubeni Nisshin Feed Co., Tokyo, Japan) (**Commercial**).

All groups except for Artemia group were fed a combination of *Artemia* nauplii and dry feed for the first 4 days of the experiment (6 – 9 dph) to ensure their survival, and then switched to dry feed (< 250 µm powder) for the rest of the experiment (10 – 26 dph). The experiment was conducted until the fish fully metamorphosed into the juvenile stage (5-26 dph). Fish were fed in excess and to ensure constant feed availability, fish were fed the feed hourly from 08:00 to 16:00. Due to its high viscosity, the dry feed was added to the surface of the tanks through a sieve (250 µm) to avoid the formation of clumps and ensure the correct particle size corresponding to the larval gape size. The tanks were siphoned twice a day to prevent deterioration of water quality due to the accumulation of waste and uneaten feed.

Sampling and Measuring

At the end of the study (26 dph), 100 fish from each tank were weighed and examined for lordosis, scoliosis, tail- and head/jaw deformities. These deformities are common in larval fish and are often considered indicators of nutritional deficiencies (Cahu et al. 2003).

Samples were also collected at the end of the study for additional analyzes. Three fish from each tank were euthanized with an overdose of MS-222 and stored in RNAlater (Invitrogen by Fisher Scientific, Waltham, WA, USA) for gut PepT1 gene expression analysis. Samples for PepT1 expression were collected 24 and 2 h after feeding to analyze postprandial and starvation-induced expression, respectively. In addition, two sets of whole-body samples were collected

from each tank, with three fish per sample. The collected fish were euthanized in liquid nitrogen (Ng and Hung, 1995) and stored at -80°C for further FAA analysis. These sample sets were collected 24 and 2 h after feeding and represented basal and postprandial FAA levels, respectively.

PepT1 Analysis

Gene expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) as described in Terova et al. (2009). Before transcript quantification of the PepT1 gene in walleye intestine, PepT1 was molecularly cloned and sequenced because the sequence was not available in the GenBank database.

Primers for amplification of a partial cDNA sequence of the PepT1 gene in Walleye were designed based on the sequence of *Sander lucioperca* (GenBank Acc. n° XM_031291729.2), Yellow Perch (*Perca flavescens*) (Acc. n° GQ906471.2), and European Sea Bass (*Dicentrarchus labrax*) (Acc. N. FJ237043.2). A strategy based on regions of strong nucleotide conservation was used to design the primers. The sequences of the primers were: 5'-TCTTCTACCTGTCCATCAATGCT-3' (Tm 59°C) for the forward primer and 5'-TTCTCCTCAGCCCAGTCCAT-3' (Tm 60°C) for the reverse primer.

For PCR amplification, an aliquot of Walleye intestinal cDNA was amplified using Taq PLATINUM SuperFi DNA Polymerase (Invitrogen) and the designed primers. The PCR thermal cycling conditions were: initial denaturation at 98°C for 30 sec, followed by 35 cycles at 98°C for 10 sec, 55°C for 10 sec, 72°C for 30 sec, and a final elongation step at 72°C for 5 min. The PCR amplification product was then loaded onto 2% agarose gel containing ethidium bromide in TAE 1X buffer to check the amplicon length. The band of interest was extracted from the gel under UV light and purified using the NucleoSpin® Gel, and PCR Clean-up Kit (Macherey-

Nagel, Germany). DNA (insert of interest) was then ligated directly to the T-tailed plasmid vector pGEM®-T Easy (Promega Milan, Italy) and subsequently sequenced in both directions (T7 and SP6). Alignment using Clustal Omega software of the partial PepT1 cDNA sequence from Walleye with PepT1 from *S. lucioperca*, *P. flavescens*, and *D. labrax* showed a high degree of similarity between the four fish species.

The RT-qPCR was performed in a final mixing volume of 20 µl containing 7 µl of Walleye cDNA (100 ng), 6 µl of iTaq Universal Probes Supermix (Bio-Rad, Milan, Italy), and 500 nM of each primer, using the CFX96 RT-PCR instrument (Bio-Rad, Milan, Italy) and following the manufacturer's instructions. The thermal reaction conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec. A blank sample containing nuclease-free water in place of the cDNA template was included as a negative control in each assay. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and β-actin as the housekeeping gene (HK). Duplicate analyses were performed for both the HK and the target gene for each sample. With respect to the HK, we tested three constitutive genes, α-tubulin, *eEF1a1*, and *β-actin,* to select the most stable one and normalize the relative quantification of PepT1 expression. Primers for the HK genes tested were designed based on the Walleye sequences of each gene available in Genbank database, whereas primers for amplification of the Walleye PepT1 gene were designed based on the partial cDNA sequence obtained by molecular cloning and sequencing mentioned above.

The accession numbers of the sequences used to design the primers for HK genes are as follows: MK860171.1 for *eEF1a1*, MK860170.1 for *α-tubulin*, and DQ231555.1 for *β-actin*. Primers were designed according to the instructions in the kit Probes Supermix. The melting temperature of the primers was 60 °C, whereas the melting temperature of the probes was at least

70°C. The open-source Primer3 program with default settings was used to design the primers and probes. To achieve the best qPCR efficiency, the amplicon length for each primer pair was set between 70 and 150 bp. The primers used for amplification of the target and HK genes are listed in Table 3.3.

Using CFX Maestro™ software (Biorad) we were able to select the best reference gene and analyze its stability using the reference gene selection tool (CFX Maestro™ Software User Guide version 1.1). After this analysis, the software indicated that both *β-actin* and eEF1α1 are the ideal HKs based on the average M value (M= 0.469) and stability value (0.758) (Figure 3.1). The efficiency of the amplification reaction is another important parameter to consider when performing relative quantification. Therefore, it was tested for each primer set using CFX Maestro™ software (Biorad). The amplification efficiency of a reaction is calculated from the following equation using data from a standard curve: Exponential Amplification = 10 (-1/slope) For this purpose, serial dilutions of Walleye cDNA were prepared and a standard curve was generated for each primer pair. Typically, the desired amplification efficiencies range from 92% to 117%. In our case, each primer set showed an amplification efficiency of more than 80% (Figure 3.2).

Free Amino Acid Analysis

Free amino acid analysis of fish tissues was performed according to Kwasek et al. (2021). Muscle samples from three fish from each tank were combined and homogenized together with 0.1 mol/L HCl at 1:9 (w/v) and spun at 12,000× g (4 °C, 15 min). Supernatants were collected, filtered (Milipore, 10-kDa cutoff at 15,000× g, 4 °C, 30 min), and then diluted with 0.1 mol/L HCl (1:19 v/v) containing norvaline and sarcosine (40 μmol/L) as internal standards. Blank samples (0.1 mol/L HCl + 40 μmol/L norvaline and sarcosine) and external standards (Sigma

acidic/neutral and basic AA) were prepared along with sample preparation. The same concentration of glutamine in 0.1 mol/L HCl as an external standard was prepared and added to the basic AA standard. Free amino acids were quantified using the Shimadzu Prominence Nexera—i LC-2040C Plus (Shimadzu, Japan) according to Shimadzu protocol No. L529 with modifications. Free amino acid concentrations (expressed as μmol/g wet body weight) were calculated in LabSolutions software version 5.92 (Shimadzu, Japan) using internal and external standards.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to visualize the products of muscle hydrolysis. Electrophoretic analysis of the samples was conducted on a discontinuous, reducing 12.5% T, SDS polyacrylamide gel (SDS-PAGE) with modifications of the method described by Updike et al. (2006). Samples were homogenized in dissociation buffer (8M urea, 2M thiourea, 60 mM Tris buffer, pH 6.8, containing 3% SDS, 350mM DDT, and 0.002% bromophenol blue) and diluted with dissociation buffer as needed. Approximately 10μl of sample (10 mg/mL) dilutions were loaded onto each lane with a 3% T stacking gel. The proteins were resolved at 150 V cm^{-1} until the dye front reached the bottom of the gel. The gel was stained with Coomassie Brilliant Blue dye (40% methanol, 5% acetic acid, 0.04% Coomassie Brilliant Blue G-250) overnight and destained with 10% acetic acid. After staining and destaining, the gel was scanned on an AZURE c600 scanner (Azure Biosystems, Dublin, CA).

Statistical Analysis

Growth performance, survival, skeletal deformity, and PepT1 expression data collected in this study were analyzed using the one-way ANOVA and a Tukey test for differences between

groups. Differences between groups were considered significant at $p < 0.05$. FAA data were analyzed using a one-way ANOVA, and differences between groups were tested using an LSD test (Fisher, 1935; Molinari et al. 2021). Differences between groups were considered significant at $p < 0.05$. Two-way ANOVAs were performed for the measured parameters between the hydrolysate-based groups (W-W, W-T, T-W, T-T) to evaluate interactions and main effects of muscle and enzyme source. Effects were considered significant at $p < 0.05$.

RESULTS

SDS-PAGE

Figure 3.3 shows the representative dietary protein profile of each experimental diet produced for this study, on a 12.5% SDS-PAGE gel. The product added into each lane contained 50% intact and 50% of the hydrolyzed protein from the corresponding muscle and enzyme combination (Lane 2: W-W; Lane 3: W-T; Lane 4: T-W; Lane 5: T-T). As neither the intact nor hydrolyzed protein from each combination were solely used in a diet, the 50/50 combination of each product best represents the molecular weight profile of the dietary protein for each diet. The method used to produce the intact and hydrolyzed products has been described and reported previously (Kwasek et al. 2021; Molinari et al. 2023).

The results of the SDS-PAGE gel show the molecular weight profiles of the dietary protein across the four muscle and enzyme combinations. Each lane containing the samples shows significant staining below 15 kDa, representing the presence of oligopeptides and possibly single peptides produced from the hydrolysis of the muscle proteins. One observable difference between the molecular weight profiles of the diets is a reduced appearance of larger polypeptides and intact proteins (> 50 kDa) in the W-T sample (Lane 3). In the case of the W-W (Lane 2), T-

W (Lane 4), and T-T (Lane 5) diets, there is strong banding that signifies the presence of these larger proteins at a range of molecular weights between 50 – 200 kDa.

Growth, Survival, and Skeletal Deformities

Based on the one-way ANOVA and Tukey test, the W-W group had a significantly higher average weight than all other groups at the end of the study. No significant differences in average weight were found between the other groups. During the study, the Artemia and Commercial groups had significantly higher survival rates than the four groups fed hydrolysate-based diets. No significant differences in survival were found among the groups fed hydrolysate-based diets, regardless of muscle or enzyme source. The results for growth and survival are shown in Table 3.4. In addition, the Commercial group had a significantly higher incidence of skeletal deformities, compared to all other groups. No significant differences were observed between the other five groups. The results for skeletal deformities are shown in Figure 3.4.

The effects of muscle and enzyme source on growth, survival, and skeletal deformities were evaluated using two-way ANOVAs for the groups that received formulated diets from the different muscle and enzyme combinations. The results of the two-way ANOVAs are shown in Table 3.5. The interaction between muscle and enzymes was significant for Walleye larval growth. No significant interaction effect on survival was observed, but muscle source had a significant effect, and the groups fed Walleye muscle-based diets had numerically lower survival. No interactions or main effects on the occurrence of skeletal deformities were observed in this study.

PepT1 Expression

There were no significant differences in PepT1 gene expression between groups 2 h and 24 h after feeding. However, PepT1 gene expression was significantly higher in the Artemia

group 2 h after feeding than 24 h after. The results for PepT1 expression are shown in Figure 3.5. The effects of muscle and enzyme source on postprandial PepT1 expression were examined using a two-way ANOVA for the groups that received formulated diets from the different muscle and enzyme combinations. The results of the two-way ANOVAs are shown in Table 3.5. In this study, no significant interactions or main effects were observed on the expression of PepT1 2 h after feeding.

Muscle Free Amino Acid Composition

The W-W group had significantly higher concentrations of total FAA and total IDAA compared with all other groups (Figure 3.6). In addition, the T-W group had significantly higher total IDAA content than the Artemia, Commercial, and T-T groups, while the content in the W-T group was significantly higher than in the Artemia group. The total DAA content was significantly higher in the Commercial and W-W groups than in the T-T group. The effects of muscle and enzyme source on the postprandial muscle FAA pool were assessed with two-way ANOVAs for the groups that received formulated diets from the different muscle and enzyme combinations. The results of the two-way ANOVAs are shown in Table 3.5. There was no significant interaction effect on 2 h postprandial total FAA, total IDAA, or total DAA levels in muscle, but both muscle and enzyme source had significant main effects on these three parameters.

Among the individual amino acids, only free aspartic acid concentrations were not significantly different between the groups. The level of free glutamic acid and free threonine in muscle was significantly higher in the Commercial group compared to the T-T group, while the level of free threonine was also significantly higher than in the Artemia group. The content of free asparagine was significantly higher in the Artemia group than in all other groups except the

Commercial group. In addition, the Artemia group had a significantly higher concentration of free serine than the W-W, T-W, and T-T groups. The free glutamine content was significantly lower in the T-T group than in the Commercial, W-W, and T-W groups. The W-W group had a significantly higher level of free histidine, glycine, and arginine in the muscle compared to all other groups, and a significantly higher level of free tyrosine than the Artemia and Commercial groups. In addition, the W-T and T-W groups had significantly higher levels of free arginine and lysine than the Artemia group. The Commercial groups had significantly higher levels of free alanine in muscle than all other groups, except the Artemia group. The free methionine content was significantly lower in the Artemia and Commercial groups than in the W-W and T-W groups, while the W-W concentration was significantly higher than in all other groups except the T-W group. Free valine concentration was significantly higher in the W-T and T-T groups than in the Artemia and Commercial groups, but significantly lower than in the W-W and T-W groups. The T-T group had significantly lower free tryptophan content than the W-W and W-T groups. The W-T groups had significantly higher free phenylalanine content than the Artemia and Commercial groups. Free isoleucine and leucine concentrations were significantly higher in the W-T group compared to all groups except the W-W group, while the T-T group also had significantly lower free isoleucine content compared to the Commercial and W-W groups. The Artemia group had a significantly lower concentration of free proline compared to all groups except the Commercial and T-T groups. Postprandial results for all FAAs analyzed are shown in Table 3.6 (2 h) and Table 3.7 (24 h).

DISCUSSION

Previous studies have compared different muscle sources for fish protein hydrolysates production (Srichanun et al. 2014; Khosravi et al. 2015; Nurdiani et al. 2016). In Asian Sea Bass

(*Lates calcarifer*), both fish muscle and squid mantle were hydrolyzed by Alcalase® and used to replace 50% of the fishmeal in the respective diets (Srichanun et al. 2014). The fish fed the fish protein hydrolysate recorded a significantly higher weight gain than the fish fed squid mantle hydrolysate (Srichanun et al. 2014). Khosravi et al. (2015) found that the addition of a krill hydrolysate significantly increased the growth of both Red Seabream (*Pagrus major*) and Olive Flounder (*Paralichthys olivaceus*), while a tuna hydrolysate had no significant effect on growth in either species. Different enzyme sources for fish protein hydrolysate production have also been tested, both commercial (Kristinsson and Rasco, 2000; Pastoriza et al. 2004; Hathwar et al. 2011) and endogenous (Aspmo et al. 2005; Silva et al. 2014; Kwasek et al. 2021; Molinari et al. 2023). Kristinsson and Rasco (2000) suggested that endogenous enzymes are more ideal for the production of protein hydrolysates. Direct comparisons between commercial and endogenous enzymes showed that extracted enzymes from fish viscera hydrolyze proteins more efficiently and achieve a higher degree of hydrolysis than the commercial enzymes used (Pastoriza et al. 2004; Silva et al. 2014).

The present study compared two different fish species as sources of muscle and endogenous enzymes to produce an optimal fish protein hydrolysate for Walleye larvae. The results of this study showed a significant increase in final weight in the W-W group compared to all other groups. Fish in the W-W group weighed 30% more than the next heaviest group (W-T) at the end of the study. The significant increase in growth compared to the Artemia and Commercial groups should be emphasized. These groups were used as a reference because they represent two current feeding systems in rearing Walleye larvae: either a commercial starter feed (Commercial; Otohime, Japan) or live feed (*Artemia* nauplii) until the fish metamorphose into juveniles (Patterson et al. 2016; Lipscomb et al. 2020). Our results show that there was a

significant interaction effect between the muscle and enzyme sources on growth, and the species-specific combination resulted in a protein hydrolysate that promoted larval growth that was higher than current feeding regimes for Walleye larvae replicated in this study.

The use of species-specific muscle as a protein source is based on the long-held theory that adult fish muscle contains the ideal amino acid profile for larvae of the same species (Cole, 1980; Wilson and Cowey, 1985). Therefore, the use of species-specific muscle in larval diets seems to be the most optimal protein source to meet the amino acid requirements for Walleye larvae growth and development. The use of hydrolyzed muscle from the same species has been tested previously (Molinari et al. 2023; Sandbakken et al. 2023). Sandbakken et al. (2023) found that partial replacement of fishmeal with a salmon hydrolysate increased the specific growth rate of Atlantic Salmon (*Salmo salar*) during the first 25 days of feeding. However, the salmon hydrolysate used in that study was compared to a fishmeal derived from multiple marine species, so there was no comparison between the salmon hydrolysate and a hydrolysate produced from another single species. The results of this study show that species-specific muscle leads to increased weight gain in Walleye larvae, and hydrolysis by species-specific enzymes clearly made the additional contribution to this significantly improved growth performance.

The use of species-specific enzymes to produce the hydrolysate was carried out to break down the protein in the same way that living fish would, but with an *in vitro* approach. Mimicking the *in vivo* digestion of the dietary protein for the larval fish served to compensate for the lack of mature enzymatic activity in the larval fish (Kolkovski, 2001). The concept of mimicking *in vivo* digestion has been used in previous studies to evaluate raw materials digestibility for various fish species (Hansen et al. 2009; Tibbets et al. 2011; Yasumaru and Lemos, 2014). Hansen et al. (2009) tested this concept specifically for predicting digestibility of

larval fish feeds using the pH-stat method, a method in which constant pH is maintained during the hydrolysis reaction by titration and the degree of hydrolysis is measured based on the amount of basic solution added during the reaction. Commercial enzymes and endogenous enzymes from Atlantic Cod (*Gadus morhua*) were utilized for that study, and only the endogenous enzymes were able to identify significant differences in digestibility between two diets tested (Hansen et al. 2009). Ultimately, the diet identified as more digestible by the Cod enzyme method significantly increased Cod larval growth (Hansen et al. 2009). Previous studies have shown that there is a significant relationship between apparent *in vivo* protein digestibility and analyzed *in vitro* digestibility of protein sources using species-specific endogenous enzymes (Tibbetts et al. 2011; Yasumaru and Lemos, 2014). The end products of these *in vitro* digestibility methods are hydrolyzed proteins that theoretically represent a dietary protein profile tailored to the digestive physiology of each specific species. In previous studies, *in vivo* digestion was mimicked in processes similar to *in vitro* digestibility tests and the hydrolyzed protein produced was used in formulated dry diets for larval fish (Kwasek et al. 2021; Molinari et al. 2023). Kwasek et al. (2021) found that the use of a muscle hydrolysate from Bighead Carp (*Hypophthalmichthys nobilis*) produced with endogenous enzymes from adult Largemouth Bass (*Micropterus salmoides*) increased the growth performance of larval Largemouth Bass. Molinari et al. (2023) tested the use of both muscle and endogenous enzymes from adult Largemouth Bass to produce hydrolysates and found that the growth of larval Largemouth Bass during the first week of feeding was significantly higher than that of larvae fed a diet based on intact protein from the same source. While this earlier study used species-specific muscle and enzymes, no comparison was made with muscle and enzymes from other species. Building on the results observed in those studies, the results of the present study demonstrate that in a direct comparison, the combination

of species-specific muscle and enzymes yields the more optimal protein hydrolysate for larval fish growth compared to muscle and enzymes from other fish species.

The mechanism behind the increase in growth in the W-W group could be a higher availability of amino acids in the diet and thus increased absorption success. Postprandial FAA results showed that Walleye muscle hydrolyzed with Walleye endogenous enzymes significantly increased the level of absorbed amino acids 2 h after feeding. The W-W group had significantly higher levels of total FAA, total IDAA, and free lysine in the muscle FAA pool than all other groups in the study. The increase in postprandial FAA likely reflects an increase in dietary amino acids available for protein synthesis. Protein synthesis in growing fish depends on a balanced FAA pool to synthesize protein and store it as new tissue (NRC, 2011). Increased dietary IDAA absorption into the FAA pool is particularly critical because these amino acids cannot be synthesized in the body. Expression of the intestinal di-/tripeptide transporter PepT1 was analyzed to gain insight into the efficiency of protein absorption from the different diets (Kwasek et al. 2012; Terova et al. 2013), but no significant differences in postprandial expression of PepT1 were observed between groups. The results of the two-way ANOVA show that neither muscle nor enzyme source had a significant effect on postprandial expression of PepT1, suggesting that the absorption levels of di-/tripeptides were similar across all the hydrolysate-based diets. Therefore, the significant differences in postprandial FAA composition of muscles in the W-W group could be due to dietary protein being absorbed in the intestine as FAA and not as di-/tripeptides. The use of species-specific sources for the preparation of fish protein hydrolysates was done to optimize the absorption and utilization of dietary protein by providing the ideal amino acid composition with muscle specifically digested for optimal absorption by endogenous enzymes. While no interaction effect was observed between muscle and enzyme

source, there were significant main effects of each source that appeared to be additive and contributed to the significant increase in FAA absorption in the W-W group.

In addition to the positive effects on growth, our study found that the addition of protein hydrolysates significantly reduced the incidence of skeletal deformities regardless of muscle or enzyme source. This was an expected result as it has been observed in many previous studies (Gisbert et al. 2012; Peruzzi et al. 2018; Kwasek et al. 2021; Molinari et al. 2023; Printzi et al. 2023). Skeletal deformities are detrimental to the aquaculture industry because deformed fish are often considered lower-grade products (Fernández et al. 2008; Shefat and Karim, 2018; Eissa et al. 2021). The occurrence of skeletal deformities has been attributed to many different nutritional deficiencies, including vitamins (Darias et al. 2011), phosphorous (Silverstone and Hammel, 2002), magnesium (Lall and Kaushik, 2021), essential fatty acids (Cahu et al. 2003), phospholipids (Coutteau et al. 1997), and amino acid deficiencies (Akiyama et al. 1986b; Shefat and Karim et al. 2018). The relationship between dietary amino acid intake and skeletal deformities was of interest in this study because species-specific muscle was selected to provide fish larvae with the ideal amino acid profile. However, the muscle source did not significantly affect the occurrence of skeletal deformities, nor did the enzyme source.

In contrast to the positive effects on skeletal development, dietary inclusion of each of the protein hydrolysates produced in this study significantly reduced survival compared to the Commercial and Artemia reference groups. The reduction in survival compared to the Artemia group was not solely due to the inclusion of protein hydrolysates, as high mortality was observed in previous studies attempting to replace live feeds with various dry feeds (Yúfera et al. 2005; Drossou et al. 2006; Lian et al. 2008). However, the lower survival rate in the hydrolysate groups compared to the Commercial group is noteworthy because the Commercial group received dry

feeds in the same quantity and duration as the hydrolysate groups. Although the differences in nutritional and physical properties between the commercial and experimental diets do not allow for a direct comparison of survival rates, the lower survival rate may be a significant barrier to the use of hydrolysates in larval diets.

The reduction in survival has been observed in previous studies examining the inclusion of hydrolysates in larval feeds (Kvåle et al. 2009; Srichanun et al. 2014; Cai et al. 2015; Molinari et al. 2023) and has been attributed to increased leaching of hydrolyzed protein (Kvåle et al. 2006; Nordgreen et al. 2009; Martínez-Alvarez et al. 2015). Leaching significantly reduces the nutritional value of feeds due to the loss of water-soluble proteins (Martínez-Alvarez et al. 2015). Protein leaching rates increase with increasing levels of hydrolysis, suggesting that more leaching occurs in diets with high levels of hydrolysates than in those with intact dietary protein (Kvåle et al. 2006; Nordgreen et al. 2009; Srichanun et al. 2014; Cai et al. 2015). Given the 37% inclusion level of the hydrolysates in this study, which accounted for 50% of dietary protein, it is likely that significant leaching from each of our hydrolysate-based diets occurred, reducing the nutritional value of the feed particles suspended in the water column. Although feed intake in this study was monitored very closely and feed was added in excess and at short intervals to ensure a constant supply of fresh food, leaching from hydrolysate-based feeds was found to occur rapidly (Martínez-Alvarez et al. 2015), with leaching of protein from small feed particles reaching its maximum withing 1-5 minutes (Kvåle et al. 2006). Thus, it is possible that some animals in the tanks that ingested the food particles more slowly and reached the maximum within 1 -5 minutes received feed that was depleted and were more susceptible to mortality than the Commercial and Artemia reference groups. This lower survival highlights the importance of optimal physical characteristics of the feed to ensure that the fish receive the full nutritional

profile of the feed. The use of binders such as guar gum and alginate can help increase pellet stability (Hardy and Barrows, 2003; NRC, 2011), reduce nutrient leaching, and further improve the future use of hydrolysate-based feeds in aquaculture.

Overall, the results of this study showed that there was a significant interaction effect between the muscle and enzyme source used to produce protein hydrolysates, and that species-specific muscle and enzymes produced a more efficient hydrolysate for larval growth compared to other combinations of muscle and enzymes. The combination of muscle and enzymes from the same-species provided an optimal amino acid profile that was broken down for efficient uptake and utilization. This species-specific combination was able to significantly increase the postprandial total FAA and IDAA content in muscle, possibly leading to increased protein synthesis and the weight gain observed in the W-W group. Future research should focus on improving the physical properties of the formulated feeds, either through binders or encapsulation techniques. This should be done to reduce possible leaching of the hydrolyzed protein in the feed and improve survival of fish larvae receiving hydrolysate-based feed.

CHAPTER 4

NEW APPROACH TO THE DEVELOPMENT OF TAILOR-MADE FEED FOR FISH LARVAE USING ZEBRAFISH *DANIO RERIO* AS A MODEL

ABSTRACT

Protein hydrolysates have been used extensively as a protein source in larval diets, but the production of these hydrolysates is typically expensive, difficult to control, and provides varying results between species when included in larval diets. This study proposed a practical controlled hydrolysis method that 1) utilizes all the endogenous enzymes contained within the fish body to "auto-hydrolyze" its tissue proteins and 2) produces a meal that is tailored to meet nutritional requirements and absorptive capacity of larval fish. The two objectives for this experiment were to determine: 1) the effect of the proposed hydrolysis method on tissue protein breakdown level; and 2) the effect of dietary inclusion of obtained hydrolysate on fish growth performance in its larval stages, using Zebrafish (*Danio rerio*) as a model. The whole bodies of adult Zebrafish were utilized in the hydrolysis process. Four Zebrafish meals were produced, three hydrolysates that were hydrolyzed for 1, 2, and 3 h, respectively, and an unhydrolyzed Zebrafish meal. The hydrolysate solution was kept at 27°C with a pH of 7-9 during the entire process. Three diets were formulated for this study, each defined by their supply of dietary protein. The Unhydro diet was solely based on the unhydrolyzed Zebrafish meal. The 50% Hydro diet was based on 50% Zebrafish hydrolysate mix and 50% unhydrolyzed Zebrafish meal. The 100% Hydro diet was 100% based on the Zebrafish meal hydrolysate. The hydrolysate mix contained equal parts of the 1, 2, and 3 h hydrolysates. Five groups were utilized in this study, with three groups receiving one of the produced Zebrafish meal-based diets. Larvae fed a commercial starter diet and *Artemia*, respectively, were also included as reference groups. At 3

days post hatch (dph), larval Zebrafish were randomly distributed into 15 (3 L) tanks, with three replicate tanks per group and 100 fish per tank. Fish were fed in excess, 10 times a day. The experiment was carried out until the fish fully metamorphosed into the juvenile stage (18 dph). The results from the SDS-PAGE showed that the proposed hydrolysis method was able to efficiently hydrolyze the protein within Zebrafish body. The results from the feeding trial found no significant differences in final weight, total length, or survival between the Unhydro, 50% Hydro, and 100% Hydro groups. The gene expression of intestinal PepT1 showed a significant increase in 50% Hydro group compared to the Unhydro group, but similar expression compared to the remaining groups in 24 h starved fish. However, 2 h after feeding, the gene expression of PepT1 showed no significant differences between 50% Hydro and the remaining groups. The 100% Hydro group had a significantly lower survival than the live feed group and showed a reduction in postprandial free amino acid levels in the muscle. These results suggest that although the hydrolysis method effectively produced hydrolyzed Zebrafish-meal, the inclusion of the hydrolysates generated the same growth of the larval Zebrafish compared to the unhydrolyzed Zebrafish-meal. These growth results paired with PepT1 gene expression potentially indicate Zebrafish larvae to be highly adapted to dry feeds at first feeding and able to utilize dietary protein in different molecular forms efficiently for growth. Overall, the proposed hydrolysis method provides a practical and cost-effective approach to producing species-specific fishmeal hydrolysates. Further research is necessary to determine whether the produced hydrolysates can improve the growth of larval fish in other fish models.

INTRODUCTION

To achieve more control over the nutritional input during the larval stage, and provide more optimal dietary protein, extensive research has centered on producing formulated diets to

reduce the use of live feeds (Cahu et al. 1998; Cahu and Infante, 2001; Koven et al. 2001; Yúfera et al. 2005; Seillez et al. 2006; Canada et al. 2017; Sheng et al. 2022). Generally, intact marine protein sources have been used in these formulated larval diets (Cahu and Infante, 2001; Langdon, 2003; NRC, 2011; Rahmdel and Falahatkar, 2021), with fish meal (FM) being the most widely used (Li et al. 2000; Nankervis and Southgate; 2006). Commercial FM is typically produced from whole fish, typically small, oily species, or wastes originating from fish processing. Fish meal is attractive as a protein source because of its high protein content, high digestibility, complete amino acid profile and high palatability (Miles and Chapman, 2006; Tacon and Metian, 2008; Salin et al. 2018). Fish meal also serves as a partial source of vitamins and minerals, and although the majority of lipids are extracted, the remaining lipids (5 – 10%) contain highly unsaturated essential fatty acids (Li et al. 2000; Hertrampf and Piedad-Pascual, 2003). Positive results on the early introduction of FM-based diets have been observed in larval rearing, with most successes occurring in co-feeding protocols where dry diets are fed along with live feed (Kolkovski et al. 1997; Roselund et al. 1997; Engrola et al. 2009).

Still, there are limitations to the ability of FM-based diets to fully replace live feeds at first feeding. For example, the complex proteins that are present in FM are not efficiently digested by larval fish especially during the first feeding stage (Conceição et al. 2010). Many fish species raised in aquaculture are altricial, not possessing fully developed digestive tracts at the start of exogenous feeding. Due to the reduced digestive capacity of altricial larval fish (Kolkovski, 2001; Tonheim et al. 2005), utilization of protein hydrolysates has been extensively studied (Kolkovski et al. 2001; Carvahlo et al. 2006; Srichanun et al. 2014; Delcroix et al. 2015; Sheng et al. 2022). Providing hydrolyzed protein in a diet for larval fish helps account for the reduced activity of proteolytic enzymes responsible for breaking down the protein to smaller

peptides and free amino acids (FAA) prior to ingestion (Chalamaiah et al. 2012). Altricial larvae have been found to have an increased assimilation efficiency of these peptides and FAA compared to intact protein (Rust et al. 1995; Tonheim et al. 2005). While the inclusion of protein hydrolysates has led to improved utilization of formulated diets in some species (Tonheim et al. 2005; Kotzamanis et al. 2007; Xu et al. 2016; Canada et al. 2019; Sheng et al. 2022), the full replacement of live feeds has remained elusive in the rearing of many commercial species.

In larval fish, amino acids are required at high levels for tissue synthesis and serve as the major source of energy (Fyhn, 1989; Parra et al. 1999; Rønnestad et al. 2003). The requirement levels for amino acids, the indispensable amino acids (IDAA) in particular, vary among species, and ages of fish within the same species (Hamre et al. 2013). While specific IDAA requirements are unknown for larval fish (Jobling, 2016), the amino acid profile of same-species muscle has been proposed to represent the amino acid requirement for larvae (Cole, 1980; Tacon and Cowey, 1985; Wilson and Cowey, 1985). Given this premise, and the known variations in amino acid profiles of FM produced from different species (Hertrampf and Piedad-Pascual, 2003), perhaps the species-specific FM obtained from adult fish would provide the right amino acid profile for fish larvae. This would be beneficial for the advancement of formulated dry diets in larval rearing, with the species-specific FM potentially serving as a dietary protein source better suited to replace live feed, compared to current commercial starter diets. Additional species-specificity can be added to incorporate the previously mentioned benefits of hydrolyzed FM, using endogenous enzymes to mimic *in vivo* digestion (Hansen et al. 2009; Tibbetts et al. 2011).

An *in vitro* hydrolysis method utilizing same-species endogenous enzymes was developed in Kwasek et al. (2021) and later tested in tandem with same-species muscle in Molinari et al. (2023). Specifically, the endogenous enzymes and muscle of adult Largemouth

Bass (*Micropterus salmoides*) were used to produce a protein hydrolysate that served as a species-specific dietary protein source for first-feeding Largemouth Bass larvae. The endogenous enzymes utilized in those studies were extracted from the harvested digestive tracts through homogenization and centrifugation, then mixed with isolated muscle. In this study, we proposed a modified and simplified method of protein hydrolysis that removes the individual extractions of muscle and digestive tracts and produces a species-specific FM from whole fish. Our hypothesis was that the use of whole fish to produce the species-specific FM would: 1) be simpler and more cost-effective than current muscle hydrolysate production methods used in aquaculture; 2) increase efficiency of hydrolysis by utilizing all the endogenous enzymes contained within the fish body to "auto-hydrolyze" its tissue proteins; and 3) provide a species-specific hydrolyzed FM that is tailor-made to match the nutritional requirements and absorptive capacity of larval fish. The objectives of this study were to; 1) determine the effect of a proposed simplified *in vitro* hydrolysis method on tissue protein breakdown level and the production of a species-specific hydrolyzed FM; and 2) evaluate the effect of dietary inclusion of the obtained FM hydrolysate on larval fish. The performance of the hydrolysates was assessed based on growth and survival, the gene expression of the intestinal peptide transporter PepT1, and the postprandial muscle FAA pool, used as an indicator of dietary amino acid availability. This study utilized Zebrafish (*Danio rerio*), as they have been found to be an ideal model species for nutritional research (Ulloa et al. 2014).

MATERIALS AND METHODS

Experimental Conditions

The feeding trial was conducted at the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University-Carbondale (SIUC), IL. The experiment was carried out

in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of SIUC. The SIUC Institutional Animal Care and Use approved all of the protocols performed (protocol #21-006). The experiment was carried out using a semi-recirculated aquaculture system (Iwaki Aquatic, Holliston, MA, USA). The system was equipped with two mechanical filters, a carbon filter, a UV light, and a biofilter. The photoperiod was maintained with overhead lights and the lights were on from 08:00 – 18:00 for 10 h of light and 14 h of darkness. The inflow was set at 100 ml/min for each tank. During the study, the temperature was 27.37^{o}C (± 0.62) and the pH was 7.25 (± 0.23). The salinity of the system was kept at 1-3 ppt to prolong the viability of the live feed (Dabrowski and Miller, 2018).

Zebrafish Hydrolysis

The hydrolysis method for this study was based on the *in vitro* method described in Kwasek et al. (2021) with modification. Briefly, adult Zebrafish were kept at 27 ^{o}C and fed to satiation one hour before harvesting. The fish were euthanized with an excess of tricaine methanesulfonate (MS-222) (Sigma-Aldrich, St. Louis, MO, USA) with a dose of 0.4 mg/mL. The whole bodies were ground three times with a meat grinder (General Food Service, Weston, FL, USA) and were stored on ice before and after each run through the grinder. After dilution with deionized water (1:2), the fish mince was homogenized with a PowerGen 1000 (Fisher Scientific, Waltham, MA, USA) tissue homogenizer on high speed for 10 min, at room temperature. The tissue to digestive tract ratio of the harvested Zebrafish was roughly 6:1. The homogenates were moved to containers (12 L), placed in a water bath at 27°C and stirred using an overhead stirrer (VWR VOS 16, VWR, Radnor, PA, USA) for the duration of the hydrolysis. The pH was adjusted to 7-9 for the entire hydrolysis to mimic the intestinal digestion of Zebrafish. Four Zebrafish meals were produced, three hydrolysates that were hydrolyzed for 1, 2,

and 3 h, respectively, and an unhydrolyzed Zebrafish meal. After the hydrolysis, the solution was brought to 90°C for 15 minutes to stop any enzymatic activity. The unhydrolyzed Zebrafish meal was immediately brought to 90°C after homogenization to prevent any hydrolysis. While this meal is not a completely intact product, it is referred to as unhydrolyzed to clearly differentiate it from the products that underwent the controlled hydrolysis process. All products were frozen at -20°C and subsequently freeze-dried to remove moisture. After freeze-drying, lipids were extracted from each product using the Soxhlet method, with ethyl ether as the solvent (Ramluckan et al. 2014). Proteomic analysis of both unhydrolyzed and hydrolyzed products was conducted at The Ohio State University, Columbus, OH.

Diets

Three diets were formulated to be isonitrogenous and isolipidic and meet the essential nutrient requirements of larval fish (NRC, 2011). They were formulated to contain 51% crude protein, and 14% crude lipids. The formulations are presented in Table 4.1. The analyzed compositions of the diets are presented in Table 4.2. The Unhydro diet did not contain any of the hydrolyzed Zebrafish meal and was solely based on the unhydrolyzed Zebrafish meal. The 50% Hydro was based on 50% Zebrafish hydrolysate mix and 50% unhydrolyzed Zebrafish meal. The 100% Hydro was 100% based on the Zebrafish meal hydrolysate. The hydrolysate mix contained equal parts of the 1, 2, and 3 h hydrolysates.

Experimental Design

At 3 days post hatch (dph), larval Zebrafish were randomly distributed into 15 (3 L) tanks, with 100 fish per tank. There were five treatment groups in this study, with three replicate tanks each. The first group, **LF**, was a live feed reference group, which received only live feed for the duration of the study. The LF group was fed rotifers (3 – 7 dph) and then switched to

Artemia nauplii (7 – 18 dph), with a transition period from 7 to 11 dph where both types of live feed were provided. The next group, **Com**, was a dry feed reference group, which received a commercial starter diet (Otohime, Marubeni Nisshin Feed Co., Tokyo, Japan). The last three groups corresponded to each of the three diets produced utilizing the Zebrafish meal. These were the **Unhydro**, **50% Hydro**, and **100% Hydro** groups The fish were fed in excess and, to ensure constant feed availability, fish received the feed every hour from 08:00 – 18:00. The size of the feeds added to the tanks were < 150 μm. The tanks were siphoned twice daily to prevent the degradation of water quality due to the build-up of waste and uneaten feed. The experiment was carried out until the fish fully metamorphosed into the juvenile stage (18 dph).

Sampling and Measuring

At the conclusion of the study (18 dph), all fish from each tank were individually weighed and the total length measured. Samples for additional analysis were also taken at the conclusion of the study. Three fish from each tank were euthanized with an overdose of MS-222 and stored in RNAlater (Invitrogen by Fisher Scientific, Waltham, WA, USA) for the analysis of PepT1 gene transcription in the gut. Samples for PepT1 expression were taken 2 and 24 h after feeding to analyze postprandial and fasting levels, respectively. Additionally, two sets of whole-body samples were taken from each tank, with five fish per sample. The sampled fish were euthanized in liquid nitrogen and stored at -80^{o}C for further FAA analysis. These sets of samples were taken 2 and 24 h after feeding and represented basal and postprandial FAA levels, respectively.

PepT1 Analysis

The digestive tracts were removed from the sampled Zebrafish. The samples were processed using TRIzol Reagent (Ambion, Foster City, CA, USA) and RNA was extracted and

purified using the On-Column PureLink DNase Treatment (PureLink™ RNA Mini Kit and PureLink DNase, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Once purified, the nanograms/µl of each RNA sample was obtained using a spectrophotometer (Nanodrop OneC, Thermo Fisher Scientific, Waltham, MA, USA). From this point, 2 µg of RNA from each sample was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) to obtain a 20 µl cDNA solution. The cDNA solutions were then added to a tube with 380 µl of water, to produce the cDNA sample for each tank. Gene expression of each cDNA sample was measured using a Bio-Rad (Hercules, CA, USA) CFX Opus 96 Real-Time PCR System. Each qPCR reaction mixture (20 µl) contained 9 µl of cDNA sample, 10 µl of PowerUp SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 0.5 µl of 800 nMol each of forward and reverse primers. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The primers are listed in Table 4.3. Each qPCR reaction was run in technical duplicates. The qPCR cycle consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 20 seconds and 60°C for 35 seconds, followed by a melting curve to ensure the amplification of only a single product in each well. Relative gene expression was calculated using the 2ΔΔCt method, normalizing the target gene expression to the expression of *ef1a* (reference gene).

Free Amino Acid Analysis

Free amino acid analysis of fish tissues was performed according to Kwasek et al. (2021). Muscle samples of three fish from each tank were combined and homogenized together with 0.1 mol/L HCl in 1:9 (w/v) and spun at 12,000× g (4 °C, 15 min). Supernatants were collected, filtered (Milipore, 10 kDa cutoff at 15,000× g, 4 °C, 30 min), and later diluted with 0.1 mol/L HCl (1:19 v/v) containing norvaline and sarcosine (40 µmol/L) as internal standards. Blanks (0.1

mol/L HCl + 40 µmol/L norvaline and sarcosine) and external standards (Sigma acid/neutral and basic AA) were prepared along with the sample preparation. The same concentration of glutamine in 0.1 mol/L HCl as an external standard was prepared and added to the basic AA standard. Free amino acids were quantified using Shimadzu Prominence Nexera—i LC-2040C Plus (Shimadzu, Japan) according to the Shimadzu protocol No. L529 with modifications. Free amino acid concentrations (expressed as µmol/g wet body weight) were calculated in LabSolutions software version 5.92 (Shimadzu, Japan) using internal and external standards.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to visualize the products of tissue hydrolysis Electrophoretic analysis of the samples was conducted on a discontinuous, reducing 12.5% T, SDS polyacrylamide gel (SDS-PAGE) with modifications of the method described by Updike et al. (2006). Briefly, samples were homogenized in dissociation buffer (8M urea, 2M thiourea, 60 mM Tris buffer, pH 6.8, containing 3% SDS, 350mM DTT, and 0.002% bromophenol blue) overnight at 25°C with agitation and diluted with dissociation buffer as needed. Approximately 10µl of sample (10 mg/mL) dilutions were loaded onto each lane with a 3% stacking gel containing 1% SDS. The proteins were resolved at 150 V cm^{-1} until the dye front reached the bottom of the gel. The gel was stained with Coomassie Brilliant Blue dye (40% methanol, 5% acetic acid, 0.04% Coomassie Brilliant Blue G-250) overnight and destained with 10% acetic acid. The destained gel was scanned on an AZURE c600 scanner (Azure Biosystems, Dublin, CA).

Statistical Analysis

The weight and total length measurements, along with survival data, were analyzed using one-way ANOVA and a Tukey test to detect significant differences between groups. The same

statistical analysis was applied to the FAA data collected, with a one-way ANOVA and a Tukey test being used to detect significant differences in FAA levels between groups within each set of samples (2 and 24 h). Lastly, the data analyzed for PepT1 expression are presented as average fold change in relation to the LF reference group, however the one-way ANOVA and Tukey test were conducted on the dCt values within each set of samples (2 and 24 h). In the case of statistical analysis conducted on all data collected, differences between groups were considered significant at $p < 0.05$.

RESULTS

SDS-PAGE

Figure 4.1 shows the results of the Zebrafish meal hydrolysates obtained utilizing 12.5% T SDS-PAGE. The products added into each lane were: Lane (1) 2 h hydrolysate; (2) broad range molecular weight standard (200–10 kDa); (3) 1 h hydrolysate; (4) unhydrolyzed; (5) 3 h hydrolysate. The gel shows that the proposed method was able to efficiently hydrolyze the protein within the Zebrafish body and produce a hydrolyzed FM. The results show that as the hydrolysis progressed, from unhydrolyzed to the 1, 2, and 3 h hydrolysates, there appear to be decreasing levels of larger protein fragments (> 50 kDa) and increasing levels of smaller proteins (< 25 kDa), and small peptides lower than 10 kDa. Evidence of hydrolysis can be observed by the disappearance of protein bands at higher molecular weights in the hydrolysate samples, that are seen in the unhydrolyzed meal. The bands are clearly seen at ~150 kDa (red asterisk), ~100 kDa (blue asterisk), and ~50 kDa (black asterisk) in Lane 4, containing the unhydrolyzed Zebrafish meal. However, the bands only appear very faintly or not at all in lanes 1, 3, and 5, which contain the varying degrees of hydrolyzed Zebrafish meal. This suggests that as the

hydrolysis progressed, the protein contained within those bands was hydrolyzed and appeared at lower molecular weights in the hydrolyzed meals.

Growth and Survival

The LF group showed significantly higher average weight and total length than the other groups. Additionally, the Com group had a significantly higher average total length than the Unhydro, 50% Hydro, and 100% Hydro groups, and a significantly higher average weight than both the 50% and 100% Hydro groups. The 100% Hydro group had a significantly lower survival, compared to the LF group. There were no significant differences in growth or survival among the Unhydro, 50% Hydro, and 100% Hydro groups. The results for growth and survival are presented in Table 4.4.

PepT1 Expression

At 24 h after feeding, the 50% Hydro group had a significantly higher expression of PepT1, compared to the Unhydro group. No other significant differences were observed between the 24 h expressions of PepT1 of the remaining groups. At 2 h after feeding, the Com group expressed significantly lower levels of PepT1 than the LF group. No other significant differences were observed between the 2 h expressions of PepT1 of the remaining groups. The results for PepT1 expression are presented in Figure 4.2.

Muscle Free Amino Acid Composition

The Com group had significantly higher postprandial levels of total FAA and free IDAA in the muscle than all other groups (Figure 4.3). The LF group had significantly higher postprandial levels of total FAA and free IDAA than the three groups that received the Zebrafish meal, and the 100% Hydro had significantly lower levels than all of the groups. Both the LF and Com groups had significantly higher postprandial levels of free dispensable amino acids (DAA)

in the muscle than the other groups, and the 100% Hydro group had significantly lower levels of DAA in the muscle, compared to the other four groups.

Significant differences were observed among the postprandial levels of individual FAA as well. The 50% Hydro group had a significantly higher level of free aspartic acid in the muscle, 2 h after feeding compared to the LF group. The postprandial levels of free glutamic acid and free arginine were significantly higher in the Com group compared to all other groups, and the LF group had significantly higher levels of both amino acids than the 3 groups that received the Zebrafish meal. The 50% Hydro group presented a significantly lower level of free asparagine than all other groups, and the Unhydro group had a level significantly lower than both the LF and Com groups. The postprandial levels of free serine and free alanine in the muscle were significantly lower in the three groups that received the Zebrafish meal, compared to the LF and Com groups. The measured levels of free glutamine were significantly higher in the LF and Com groups, compared to all other groups, and the Unhydro group had a significantly higher level than the 100% Hydro group. Significant differences between each group were observed for both free histidine and free glycine levels in the muscle. The highest levels were observed in the Com group, followed by the LF, Unhydro, 50% Hydro, and 100% groups, respectively. The 100% Hydro group had significantly lower levels of free threonine than all other groups, and the Com group had significantly higher levels than all other groups. The LF group had a significantly higher level of free tyrosine in the muscle 2 h after feeding than all other groups, and the Com group also had a significantly higher level than the three Zebrafish meal groups. The Unhydro group had a significantly higher level of free tyrosine than the 50% and 100% Hydro groups. The postprandial levels of free lysine, free methionine, and free valine were significantly higher in the Com group compared to all other groups, and the levels of all three amino acids were

significantly higher in the LF group compared to the Unhydro, 50% Hydro, and 100% Hydro groups. For free lysine, the level was significantly lower in the 100% Hydro group compared to the Unhydro group. The level of free methionine was significantly higher in the Unhydro and 50% Hydro groups than the 100% Hydro group. The 50% Hydro group had a significantly higher level of free valine, compared to the 100% Hydro group. Both the LF and Unhydro groups had significantly lower levels of free tryptophan in the muscle, compared to the Com group. The level of free phenylalanine in the muscle 2 h after feeding was significantly higher in the Com group than all other groups. The Com group also had significantly higher levels of free isoleucine and free leucine, compared to all other groups. Both the LF and 50% Hydro groups had significantly higher levels of free isoleucine than the 100% Hydro group. The 100% Hydro group showed a significantly lower level of free leucine, compared to the Unhydro and 50% Hydro group. Lastly, the LF group had a significantly higher level of free proline in the muscle than all other groups, and the Com group had a significantly higher level than the three groups that received the Zebrafish meals. The 100% Hydro group had a significantly lower level of free proline than all other groups. The results for all postprandial levels of FAA analyzed are presented in Table 4.5 (2 h) and Table 4.6 (24 h).

DISCUSSION

There were two main objectives in this study, the first being to assess the efficacy of the proposed hydrolysis method on tissue protein breakdown level and the production of a species-specific hydrolyzed FM. The efficacy of the hydrolysis process was visualized with an SDS-PAGE, showing the molecular weight profile of the protein over the progression of the incubated hydrolysis process. The proteomic results show that the method was able to hydrolyze the tissue proteins in the Zebrafish body and produce protein/peptide products with a higher proportion of

low molecular weight proteins than the unhydrolyzed product. The increased proportion of these low molecular weight proteins/peptides was intended to provide dietary protein that would be more efficiently absorbed by the larval fish. Studies suggest that small peptides ranging from 5 – 75 kDa are more efficiently absorbed by larval fish than larger, intact proteins (Tonheim et al. 2005; Canada et al. 2017). Based on electrophoretic analysis, there appears to be more of these smaller peptides in the hydrolyzed Zebrafish meals, compared to the unhydrolyzed meal, with increasing amounts of peptides < 10 kDa appearing as the time of hydrolysis progressed.

The major benefits of the proposed hydrolysis method are rooted in its simplicity, reproducibility, and species-specificity. The autolytic basis of this hydrolysis method is similar to that utilized in the production of fish silage. In traditional silage production, fish by-products or whole fish are minced, and the tissue is allowed to be autolyzed by proteolytic enzymes contained in both the guts and muscle (Liaset et al. 2000; Vidotti et al. 2003; Hardy, 2008; Olsen and Toppe, 2017). To maintain ideal pH for silage, and prevent microbial growth, organic or mineral acids are added to the silage solution (Ghaly et al. 2013). During the silage process, the endogenous enzymes break down the tissue proteins to produce a liquid with high levels of water-soluble peptides and FAA (Kristinsson and Racsco, 2000). Silage production represents a cost-effective and simple way to produce hydrolyzed fish protein. However, this process can be time consuming and presents significant variations in protein recovery and degree of hydrolysis (Espe and Lied, 1999; Liaset et al. 2000). As a result, recent research has focused on enzymatic hydrolysis as a method of producing fish protein hydrolysates. Enzymatic hydrolysis involves the addition of separately obtained proteolytic enzymes to hydrolyze the protein in an incubated system (Kristinsson and Rasco, 2000; Siddik et al. 2021). This method provides more control over the peptide profile of the hydrolysate, with the selection of enzymes being based on their

cleavage-specificity (Ryu et al. 2021). There is also greater control over the degree of hydrolysis as the pH and temperature of the method can be adjusted throughout the process to maintain an optimal environment for the activity of the selected enzymes (Siddik et al. 2021). This ultimately results in a higher degree of hydrolysis in a shorter amount of time, and a more consistent and reproduceable protein profile than silage production (Ryu et al. 2021; Siddik et al. 2021). A major pitfall to the use of enzymatic hydrolysis is the difficulty in selecting the most ideal enzyme(s) and their high cost (Wisuthiphaet et al. 2015; Olsen and Toppe, 2017).

The hydrolysis method in this study provides a middle ground between these two production methods, with added benefits of species-specific protein and species-specific enzymes. In our method, whole fish were minced, and the proteins were autolyzed using only the endogenous enzymes. The autolysis was continuously monitored and controlled to maintain a constant temperature and pH, mimicking the *in vivo* digestion of Zebrafish digestive tract. While the digestive enzymes play the most significant role in the autolysis process in fish, other enzymes contained within the body have proteolytic abilities and contribute to breakdown of body proteins (Kristinsson and Rasco, 2000; Ghaly et al. 2010). Lysosomes within fish tissues such as the muscle, kidney, and liver contain proteases, including cathepsins, that assist in the hydrolysis of tissue proteins (Mukundan et al. 1986; Kristinsson and Rasco, 2000; Ghaly et al. 2010). Additionally, aminopeptidases contained within the intestinal epithelial layer (German et al. 2010), and alkaline phosphatase present in many other fish tissues (Gisbert et al. 2018) likely played a significant role in the hydrolysis process, as those enzymes are most active at an alkaline pH.

Additionally, the *in vitro* hydrolysis method of mimicking *in vivo* digestion with endogenous enzymes has been proven to be effective in this and other studies (Silva et al. 2014;

Moyano et al. 2015; Kwasek et al. 2021). This mimicked *in vivo* digestion produces a dietary protein that is hydrolyzed with the species-specific composition of proteolytic enzymes, representing a molecular weight profile that likely matches that of protein digested within the fish body. It also produces a FM that provides the optimal amino acid profile for the larvae of the same species (Akiyama et al. 1997; Meyer and Fracalossi, 2005). The continuous control over pH and temperature of this method allows it to be applicable to other species as a means of producing species-specific FM. Although in the present study the pH was maintained to be constantly alkaline to mimic the intestinal digestion of Zebrafish, an agastric species, the same general incubation method was utilized in a gastric species (Kwasek et al. 2021). That study switched the pH of the hydrolysis solution from acidic to alkaline to mimic the movement from stomach (acidic digestion) to intestine (alkaline digestion), and found that the activity of the endogenous enzymes, and thus hydrolysis of the protein, continued after the pH switch (Kwasek et al. 2021). The proposed method provides a practical way to produce hydrolyzed, species-specific FM, where the cost-effectiveness, simplicity, and autolysis aspects of silage production are maintained, and the efficiency and control are improved through monitored incubation.

Another benefit of the presented hydrolysis method is that it produces a hydrolyzed FM out of whole-body fish, instead of just isolated muscle. Not only does this contribute to the simplicity of the method by removing the dissection and isolation of muscle and digestive tracts, but it also provides a more sustainable and complete dietary source. The harvesting of muscle from fish, or filleting fish, leaves behind 50 – 70% as waste (Rustad et al. 2011; Siddik et al. 2021). The fillet from fish contains only 15-25% of the total protein in the body (Heffernan et al. 2021; Ryu et al. 2021) and leaves behind a fish frame that contains the head, tail, scales, bones, and viscera (Ghaly et al. 2013). These often-discarded parts of the fish contain ~58% protein

(Ghaly et al. 2013) and can provide additional nutrients including minerals, vitamins, and essential fatty acids (Jobling et al. 2001; Jayathilakan et al. 2012; Petricorena, 2015). Although the levels and effects of these components were not directly assessed in this study, further investigation and modifications to this method could present the potential of the hydrolyzed FM to be a species-specific source of these nutritional components as well. In an industry where future growth hinges on increased sustainability, a novel method that discards over 50% of its materials as waste would be short-lived. Thus, the use of whole fish as a source of highly digestible protein/peptides in FM as described in this study presents a more viable and environmentally conscious approach to produce a species-specific diet for larval fish.

The second main objective of this study was to assess the effect of dietary inclusion of the obtained FM hydrolysate on larval fish. The sole use of the unhydrolyzed meal and hydrolyzed Zebrafish meal mix was tested alongside the 50/50 combination of both. The LF and Com groups served as benchmarks for growth and physiological responses of Zebrafish under typical commercial rearing methods. Only the 100% Hydro group exhibited a significant difference in survival, significantly lower than the LF group. In relation to the Com group, all three of the Zebrafish meal groups showed a significantly lower total length, and the 50% Hydro and 100% Hydro groups exhibited significant reductions in average weight. While the Com group provides a reference for larval rearing on available commercial starter diets, important variables like dietary composition, ingredients, and physical structure are not controlled for, which limits the ability to make strong conclusions on the dietary effects behind the differences observed. Thus, the most informative comparisons in this study come from the three groups fed with the formulated Zebrafish meal-based diets.

The growth and survival results from this study showed no significant differences in weight, total length, or survival between the Unhydro, 50% Hydro, and 100% Hydro groups. This lack of improvement through the inclusion of hydrolyzed protein contradicts results observed in many other larval studies (Kotzamanis et al. 2007; Srichanun et al. 2014; Kwasek et al. 2021; Sheng et al. 2022; Molinari et al. 2023). As mentioned previously, the inclusion of the hydrolyzed Zebrafish meal in the diet was intended to promote increased growth by providing protein in the form of small peptides and FAA. The dietary provision of these small peptides and FAA has been found to increase absorption of dietary protein by larval Walleye (*Sander vitreus*) (Rust, 1995, Chapter 3 above), and Striped Bass (*Morone saxatilis*) (Rust, 1995), as well as larval Atlantic Halibut (*Hippoglossus hippoglossus*) (Kvale et al. 2007), and Senegalese Sole (*Solea senegalensis*) (Canada et al. 2017). The absorption of the di-/tripeptides from the diets was assessed based on the expression of PepT1, an intestinal peptide transporter (Verri et al. 2003; Terova et al. 2013). PepT1 expression has been found to be responsive to different molecular weight profiles of dietary protein (Ostaszewska et al. 2010; Kwasek et al. 2012; Sheng et al. 2023).

At the 2 h sampling time, there was a numerical trend of lower PepT1 expression in the Unhydro, 50% Hydro, and 100% Hydro groups, and a significant downregulation in the Com group compared to the LF group. This difference between live feed and dry feed groups highlights a major benefit of live feeds. Live feeds contain high levels of water-soluble protein in low molecular weight form, which makes the dietary protein more bioavailable than that in formulated dry diets (Holt, 2011; Hamre et al. 2013). This may have resulted in a faster absorption of di-/tripeptides in the LF group and a delayed expression of PepT1 in the dry feed groups. This delay in PepT1 expression has been observed in Rainbow Trout (*Oncorhynchus*

mykiss) as well, where the expression of PepT1 was significantly higher 12 h postprandially, compared to 2 h in response to dry diets (Borey et al. 2016). Consequently, the 2 h postprandial results showed no significant differences in PepT1 expression between the Unhydro, 50% Hydro, and 100% Hydro groups. However, 24 h after feeding, there was a significant upregulation in PepT1 expression in the 50% Hydro group over the Unhydro group. This might suggest that the 50% inclusion of the hydrolyzed Zebrafish meal significantly increased the absorption of dietary protein in di-/tripeptide form. Previous studies that have shown increases in PepT1 expression in response to dietary peptides also observed these differences at 24 h post-feeding (Cai et al. 2015; Wu et al. 2018; Sheng et al. 2023). In larval Snakehead (*Channa argus*), the 50% inclusion of a protein hydrolysate led to a significant increase in the 24 h PepT1 expression over a diet containing no protein hydrolysates and a diet containing 100% of its dietary protein in hydrolyzed form (Sheng et al. 2023). These results are similar to the 24 h PepT1 results in the present study, although there was only a numerical decrease in PepT1 expression in our 100% Hydro group compared to the 50% Hydro group. In the larval Snakehead, the significant increase in PepT1 correlated with a significantly higher growth due to the increased absorption of di-/tripeptides (Sheng et al. 2023), however in the present study, there were no significant differences in growth between the Unhydro, 50% Hydro, and 100% Hydro groups. The lack of significant differences in growth between these three groups could suggest that Zebrafish are highly adapted to dry feeds at first feeding and are able to utilize dietary protein in different molecular forms efficiently for growth. The adaptation to dry feeds may stem from a high level of domestication in Zebrafish.

Domestication in fish has been found to influence the digestive abilities of larvae (Chen et al. 2017; Palińska-Żarska et al. 2020; Montero et al. 2023). In the case of Eurasian Perch

(*Perca fluviatilis*), a popular aquaculture species in Europe, larvae produced from domesticated broodstock were directly compared with larvae from wild-caught broodstock (Palińska-Żarska et al. 2020). The researchers compared growth rates and digestive enzyme activities of these larvae, and found that once compound diets were introduced, the domesticated larvae started showing significantly higher growth rates compared to the wild-type larvae (Palińska-Żarska et al. 2020). This increase in growth rate corresponded with an overall decrease in the activity of most digestive enzymes, but an increase in proteolytic enzymes in the domesticated larvae (Palińska-Żarska et al. 2020). This difference in digestive enzyme activity was suggested to be a result of "nutritional monotony", where generations of domesticated Eurasian Perch were fed with high-protein compound diets, and consequently had undergone a shift towards higher proteolytic activity to digest those diets (Palińska-Żarska et al. 2020). An increase in proteolytic enzyme activity was also observed in the offspring of domesticated Gilthead Seabream (*Sparus aurata*) (Montero et al. 2023). The offspring showed significantly higher levels of both pepsin and chymotrypsin, compared to the wild-type reference group (Montero et al. 2023). These studies provide evidence that domestication in fish species can affect the abilities of larvae to digest and utilize dietary components from formulated feeds over generations.

Additional evidence of the effect of possible Zebrafish domestication on the results in this study and other larval studies may be found in the high survival rates achieved with full live feed replacement. The Unhydro and 50% Hydro diets were able to fully replace live feed in this study without significant reductions in survival, supporting survival rates ~76%. This full replacement of live feed has previously been achieved in the rearing of larval Zebrafish with similar survival levels (Carvahlo et al. 2006; Kaushik et al. 2011; Farias and Certal, 2016; Printzi et al. 2023). Compared to the low survival levels of complete live feed replacement in

commercial species like Largemouth Bass (Molinari et al. 2023), Barramundi (*Lates calcarifer*) (Curnow et al. 2006), Sea Bass (*Dicentrarchus labrax*) (Cahu et al. 1998), and Senagalese Sole (Canavate and Fernández-Díaz, 1999), the observed success of dry feed-only rearing for Zebrafish presents a stark contrast. The significant difference in survival between Zebrafish and those other commercial species could be due to the varying levels of domestication. Domesticated fish have exhibited higher feed intake and reduced stress in intensive settings, contributing to improved survival over their wild-type counterparts during intensive rearing (Robison and Rowland, 2005; Chen et al. 2017; Nen et al. 2018; Milla et. 2021). Zebrafish are a popular ornamental and lab-raised model species and are classified at the highest level of domestication (Balon, 2004; Teletchea, 2016). Common Carp (*Cyprinus Carpio*) is another cyprinid species within this same level of domestication that has shown high survival of larvae reared on dry diets only (Carvahlo et al. 1997). Meanwhile, most commercial species in aquaculture exist at lower levels of domestication (Teletchea, 2021). For Carp, this increased domestication is a result of a longer history of culture compared to other commercial species. In contrast, Zebrafish have been commercially cultured for much less time, but still reached the highest domestication level. This is a result of very short generation times in Zebrafish (~2 - 4 months) (Lawrence et al. 2012), which has allowed for genetic changes related to domestication to occur much more rapidly compared to other commercialized species (Suurvälli et al. 2020). Thus, the varying degrees of domestication among species may be a factor behind the differences in survival between the live feed replacement studies.

Another plausible reason behind the lack of differences in growth across the Unhydro, 50% Hydro, and 100% Hydro groups, is that the dietary protein profiles of each diet were more similar than they were formulated to be. The Unhydro diet was formulated to provide a dietary

protein profile with larger, intact fragments, while the 100% Hydro diet was formulated to provide dietary protein in the form of low molecular weight peptides and FAA from the hydrolyzed Zebrafish meal. The 50% Hydro diet was formulated to be a middle ground between those diets, with half of its dietary protein supplied from the unhydrolyzed Zebrafish meal, and the other half from the mix of hydrolyzed Zebrafish meal. Looking at the results from the SDS-PAGE, it is feasible that our "unhydrolyzed" Zebrafish meal may have undergone unintended and unincubated partial hydrolysis. While the unhydrolyzed Zebrafish meal does contain a higher level of large protein fragments > 25 kDa compared to the hydrolyzed products, there seems to be a fair amount of smaller proteins < 25 kDa present. These small fragments may be a result of unintended autolysis occurring during storage prior to and during the homogenization of whole fish. While the fish were stored on ice during harvest, reducing the post-harvest hydrolysis rate (Tamotsu et al. 2018), the water used during homogenization was room-temperature. This could have allowed for the enzymes contained within the body to partially hydrolyze the body protein during the 10-minute homogenization at room temperature. A partial hydrolysis of our 'unhydrolyzed' Zebrafish meal could have reduced the variations in dietary molecular weight profiles among our formulated experimental diets. This likely contributed to reduce differences in growth from occurring among the Unhydro, 50% Hydro, and 100% Hydro diets. This poses a limitation to this method, and further research to prevent unintended hydrolysis from occurring prior to incubation would improve control over the final degree of hydrolysis.

The postprandial levels of FAA in the muscle were significantly lower in the 100% Hydro group, compared to the other groups. While this reduction in postprandial FAA has typically been attributed to excess leaching from hydrolysate-based diets (Langdon, 2003; Kvåle et al. 2007; Nankervis and Southgate, 2009), it is unlikely that this led to the reduced FAA levels

in the 100% Hydro group. Precautions like smaller meals in shorter time intervals and 2x daily cleanings were taken to ensure pellets consumed by the larvae had not experienced prolonged exposure in the water column. Additionally, no significant differences in growth or survival were seen among the Zebrafish-meal fed groups, suggesting any potential leaching differences among diets did not significantly affect larval performance. However, the 100% Hydro group was the only group to show a significant reduction in survival relative to the LF group. Previous studies have found that high inclusion levels of hydrolysates have hindered the utilization of dietary protein in larval fish, leading to a rapid absorption and excretion of amino acids, or an oversaturation of FAA transporters that limited absorption (Cahu et al. 2004; Liu et al. 2006; Canada et al. 2019). The supply of amino acid from the diet is critical to support the high levels of tissue synthesis required during larval metamorphosis (Hamre et al. 2013). Given the significantly reduced survival and postprandial FAA levels, it is likely that the full supply of dietary protein in hydrolyzed form reduced dietary amino acid absorption and utilization to a point that hindered the proper development of the larval Zebrafish and led to a significant decrease in survival of the 100% Hydro group.

Overall, the results from this study showed that the proposed hydrolysis method was able to efficiently hydrolyze the protein within Zebrafish body. This provides a practical, cost-effective, and sustainable method for producing hydrolyzed species-specific FM, that is applicable to a wide range of species. Results from the feeding trial show that the inclusion of the hydrolysates generated the same growth of the larvae compared to the Unhydrolyzed Zebrafish meal. The growth results paired with Pept1 gene expression potentially indicate Zebrafish larvae are highly adapted to dry feeds at first feeding and able to utilize dietary protein in different molecular forms efficiently for growth.

CHAPTER 5

THE EFFECT OF DIETARY INDISPENSABLE AMINO ACID DEFICIENCY ON FEEDING RESPONSE IN STOMACHLESS FISH

ABSTRACT

The evidence suggests that fish seem to be more tolerant than mammals to imbalanced dietary amino acid profiles. However, the behavioral and physiological responses of fish to individual deficiencies in dietary indispensable amino acids (IDAA) remain unclear. The objective of this study was to determine how stomachless fish respond to diets deficient in the main limiting IDAA (lysine, methionine, and threonine), using Zebrafish (*Danio rerio*) as a model species. The response to the deficient diets was assessed based on; 1) growth performance; 2) feed intake; 3) the expression of appetite-regulating hormones and nutrient-sensing receptors, and 4) muscle postprandial free amino acid (FAA) levels. Six semi-purified diets were formulated for this study. The CG diet contained casein and gelatin as its only protein sources, while FAA50 diet had 50% of is dietary protein supplied with crystalline amino acids. Both were formulated to contain identical, balanced amino acid profiles. The remaining diets were supplied with the same amino acid mix as the FAA50 diet, but with minor adjustments to create deficiencies of the selected IDAA. The (-) Lys, (-) Met, and (-) Thr diets had lysine, methionine, and threonine withheld from the FAA mix, respectively, and the Def diet was deficient in all three. At 21 days post hatch (dph), juvenile Zebrafish were randomly distributed into 18 (3 L) tanks, with 30 fish per tank. There were six treatment groups in this study, with three replicate tanks each. Each group corresponded with one of the six formulated diets and received only the specified diet for the duration of the experiment. The fish were fed to apparent satiation three times a day, and each feeding was carefully observed to ensure all feed added to

the tanks was consumed. The experiment was carried out until 50 dph. The results from this study found that the 50% replacement of dietary protein with crystalline amino acids significantly reduced the growth of juvenile Zebrafish. There were no significant differences in growth between the FAA50 group and any of the groups that received a deficient diet. The deficiency of singular IDAA did not induce a significant change in feed intake, however, the combined deficiency of all three in the Def diet led to a significant increase in feed intake. This increased feed intake also led to a decrease in feeding efficiency, which was also observed in the (-) Lys group. There was also an observed upregulation of *neuropeptide Y* (*NPY*), an orexigenic hormone, in the Def group, compared to the FAA50 group. The overall results from this study suggest stomachless fish increase their feed intake when challenged with IDAA-deficient diets, and that the regulation of NPY might play a role in this response. Further research is required to determine the direct pathway behind the upregulation of *NPY*, or to identify other receptors or hormones involved in the response to dietary IDAA deficiencies.

INTRODUCTION

The amino acid profile of the dietary protein is critical to the proper development and physiological performance of fish. The 20-standard protein-bound amino acids are divided into two major categories, indispensable and dispensable. Indispensable amino acids (IDAA) are unable to be synthesized within the body and therefore, must be obtained from the diet. Dispensable amino acids (DAA) can be synthesized within the body using IDAA; therefore, they do not have explicit individual requirement levels in the diets (NRC, 2011). These amino acids and their metabolites play significant roles in many physiological processes including immune response (Li and Gatlin, 2006; Li et al. 2007), neurological function and development (Bordieri et al. 2005), growth and feed efficiency (Yan and Qiu-Zhou, 2006), stress response (Lepage et al.

2002; Damasceno-Oliveira et al. 2007), and osmoregulation (Li et al. 2009). Additionally, the proper balance of these amino acids within the diet is critical to the successful utilization of the dietary protein in fish and efficient protein tissue accretion (Hamre et al. 2013). Indispensable amino acids that are significantly deficient in the diet can become limiting amino acids. In fish, the first three limiting amino acids are lysine, methionine, and threonine (Li et al. 2009; Yaghoubi et al. 2017). Limiting amino acids set the ceiling on protein synthesis, as proteins can only be synthesized based on the availability of the most limiting amino acid and other dietary amino acids will be wasted (catabolized and/or excreted) as a result (Hou and Wu, 2018). Thus, diets deficient in IDAA can have negative impacts on fish and their dietary protein utilization. Dietary amino acid deficiencies lead to amino acid losses, and increased deamination of amino acids for physiological processes other than protein synthesis (Ballantyne, 2001). The reduction in protein synthesis can then lead to a significant decrease in growth and many health consequences, like skeletal deformities (Prabu et al. 2017). In a study done on juvenile Midas (*Amphilophus citrinellum*), a diet deficient in multiple IDAA led to a significantly reduced weight gain and survival (Dabrowski et al. 2007). Gómez-Requeni et al. (2003) found that a diet with a low IDAA/DAA ratio significantly increased the feed conversion ratio of Seabream (*Sparus aurata*) and reduced nitrogen retention.

However, how fish respond behaviorally to diets with IDAA deficiencies still remains unclear. The fish could reject the diet due to the deficiency and reduce feed intake; or they could increase feed intake to compensate for the existing deficiency. The reduction in feed intake as a response has been extensively studied in mammals (Montgomery et al. 1978; Nam et al. 1995; Koehnle et al. 2003; Gloaguen et al. 2012). The ingestion of a valine deficient diet in pigs resulted in a ~14% reduction in feed intake after just one hour (Gloaguen et al. 2012). In rats the

response is even more rapid, these animals will reduce their feed intake of diets lacking IDAA within just minutes of exposure (Koehnle et al. 2003). The behavioral responses have also been studied in fish, although less extensively and with mixed results. Yamamoto et al. (2001) found that Rainbow Trout (*Oncorhynchus mykiss*) were able to detect insufficient levels of dietary lysine and as a result, reduced their intake of that diet. Additionally, feed intake in Silvery-black Porgy (*Sparidentex hasta*) was significantly reduced in response to deficiencies in all IDAA, except arginine (Yaghoubi et al. 2017). In contrast, Dabrowski et al. (2007), observed juvenile Midas significantly increased their feed intake of diets that were deficient in multiple IDAA after two weeks of feeding.

Based on the available evidence, fish seem more tolerant than mammals to imbalanced dietary amino acid profiles, however, this hypothesis is based on minimal fish studies, with only few species tested. To gain a clearer picture of the behavioral response of fish towards IDAA deficiencies, specifically towards deficiencies of singular IDAA, we utilized Zebrafish (*Danio rerio*) as the experimental species. Zebrafish have been considered an ideal model in nutrition (Ulloa et al. 2014) and are closely related to other cyprinids utilized in studies looking at dietary free amino acids (FAA) (Dabrowski et al. 2007; Kwasek et al. 2010). The objective of this study was to determine how fish respond to diets deficient in the main limiting IDAA (lysine, methionine, and threonine), and how those diets impact; 1) growth performance; 2) feed intake; 3) the expression of appetite-regulating hormones and nutrient-sensing receptors, and 4) muscle postprandial FAA levels.

MATERIALS AND METHODS

Experimental Conditions

The feeding trial was conducted at the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University-Carbondale (SIUC), IL. The experiment was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of SIUC. The SIUC Institutional Animal Care and Use approved the protocols performed (protocol #21-006). The experiment was carried out using a semi-recirculated aquaculture system (Iwaki Aquatic, Holliston, MA, USA). The system utilized two mechanical filters, a carbon filter, a UV light, and a biofilter. The overhead lights were on from 08:00 – 18:00 to maintain a photoperiod of 10 h of light and 14 h of darkness. The illumination provided was at 245 lux and the distance between the surface of the water and the light source was 10 cm. The inflow was set at 200 ml/min for each tank. During the study, the temperature was 27.50^{o}C (± 0.31) and the pH was 7.17 (± 0.19).

Diets

Six semi-purified diets were formulated to be isonitrogenous and isolipidic and meet the essential nutrient requirements of juvenile cyprinids (NRC, 2011). The requirement levels for IDAA were based of those listed for Common Carp (*Cyprinus carpio*), and were 2.2% lysine, 0.7% methionine, and 1.5% threonine (NRC, 2011). The diets were formulated to contain 49% crude protein and 10% lipids and were formulated similarly to previous semi-purified diets tested on other cyprinid species (Dabrowski et al. 2007; Ostaszewska et al. 2008; Kwasek et al. 2009; Kwasek et al. 2010). The formulations are presented in Table 5.1. The first diet, CG, contained casein and gelatin as its only protein sources. The remaining five diets had 50% of its protein supplied through casein and gelatin, and the other 50% supplied in the form of a FAA. The

FAA50 diet was supplied with an FAA mix formulated to create a diet that matched the AA profile of the CG diet. The other four diets were supplied with the same FAA mix as the FAA50 diet, but with minor adjustments to create deficiencies of the selected IDAA. The (-) Lys, (-) Met, and (-) Thr diets had lysine, methionine, and threonine withheld from the FAA mix, respectively. Finally, the Def diet was FAA50 diet deficient in all three of the selected amino acids. To keep the nitrogen levels consistent between diets, glycine was supplemented in place of the withheld amino acids. The composition of the FAA mixes used in each diet is presented in Table 5.2. The analyzed composition of the diets are presented in Table 5.3.

Experimental Design

At 21 days post hatch (dph), juvenile Zebrafish were randomly distributed into 18 (3 L) tanks, with 30 fish per tank. There were six treatment groups in this study, with three replicate tanks each. Each treatment corresponded with one of the six formulated diets and received only the specified diet for the duration of the experiment. There were three feedings a day and during each feeding the fish were fed to apparent satiation, meaning the fish were fed until the cessation of feeding behavior was observed. At the start of each feeding, small amounts of feed were added to each tank and once consumed, more feed was added. This process was repeated until more than 85% of the fish in the tank had stopped consuming the feed. The feedings were carefully observed to ensure all feed added to the tanks was consumed. The feed containers were weighed at the end of each day to measure the daily feed intake. The daily feed intake was assessed based on the total feed consumed in each tank divided by the number of fish in the tank on that day to account for mortalities that occurred during the trial. The experiment was carried out until 50 dph.

Sampling and Measuring

At the conclusion of the study (50 dph), all fish from each tank were individually weighed. Based on the weight gain measured for each group, the feeding efficiency was calculated by taking the weight gain divided by the feed intake. Samples for additional analysis were also taken at the conclusion of the study. Three fish from each tank were euthanized with an overdose of tricaine methanesulphonate (MS-222), and the heads and digestive tracts were harvested and stored at -80^{o}C for the analysis of gene expression. These samples were taken 24 h after the final feeding. Additionally, two sets of muscle samples were taken from each tank, with three fish per sample. The sampled fish were euthanized in liquid nitrogen and stored at -80^{o}C for further FAA analysis. These sets of samples were taken 2 and 24 h after feeding and represented basal and postprandial FAA levels, respectively.

Hormone/Gene Expression Analysis

The digestive tracts and brains were dissected from the sampled Zebrafish. The samples were processed using TRIzol Reagent (Ambion, Foster City, CA, USA) and RNA was extracted and purified using the On-Column PureLink DNase Treatment (PureLink™ RNA Mini Kit and PureLink DNase, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Once purified, the nanograms/µl of each RNA sample was obtained using a spectrophotometer (Nanodrop OneC, Thermo Fisher Scientific, Waltham, MA, USA). From this point, 2 µg of RNA from each sample was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) to obtain a 20 µl cDNA solution. The cDNA solutions were then added to a tube with 380 µl of water, to produce the cDNA sample for each tank. Gene expression of each cDNA sample was measured using a Bio-Rad (Hercules, CA, USA) CFX Opus 96 Real-Time PCR System. Each qPCR reaction mixture (20 µl) contained 9 µl

of cDNA sample, 10 µl of PowerUp SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 0.5 µl of 800 nMol each of forward and reverse primers. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primers were obtained for *orexin*, *leptin*, *cholecystokinin* (*CCK*), *ghrelin*, *neuropeptide Y* (*NPY*), G protein-coupled receptor class C group 6 member A (*gprc6a*), calcium-sensing receptor (*casr*), and taste-receptor type 1 member 2 (*tas1r2*). The primers are listed in Table 5.4. Each qPCR reaction was run in technical duplicates. The qPCR cycle consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 20 seconds and 60°C for 35 seconds, followed by a melting curve to ensure the amplification of only a single product in each well. Relative gene expression was calculated using the 2ΔΔCt method, normalizing the target gene expression to the expression of *ef1a* (reference gene). The FAA50 group was utilized as the control group to measure a relative change in expression based solely on the deficiencies of the IDAA, thus the CG group not used in this analysis.

Free Amino Acid Analysis

Free amino acid analysis of fish tissues was performed according to Kwasek et al. (2021). Muscle samples of three fish from each tank were combined and homogenized together with 0.1 mol/L HCl in 1:9 (w/v) and spun at 12,000× *g* (4 °C, 15 min). Supernatants were collected, filtered (Milipore, 10 kDa cutoff at 15,000× *g*, 4 °C, 30 min), and later diluted with 0.1 mol/L HCl (1:19 v/v) containing norvaline and sarcosine (40 µmol/L) as internal standards. Blanks (0.1 mol/L HCl + 40 µmol/L norvaline and sarcosine) and external standards (Sigma acid/neutral and basic AA) were prepared along with the sample preparation. The same concentration of glutamine in 0.1 mol/L HCl as an external standard was prepared and added to the basic AA standard. Free amino acids were quantified using Shimadzu Prominence Nexera—i LC-2040C

Plus (Shimadzu, Japan) according to the Shimadzu protocol No. L529 with modifications. Free amino acid concentrations (expressed as μmol/g wet body weight) were calculated in LabSolutions software version 5.92 (Shimadzu, Japan) using internal and external standards.

Statistical Analysis

The growth performance, survival, feeding parameters, and FAA data were analyzed using one-way ANOVA and a Tukey test to test differences between groups. Differences between groups were considered significant at p values < 0.05. The gene expression data was analyzed using a one-way ANOVA and differences between groups were tested using an LSD test (Fisher, 1935) (Molinari et al. 2021). Differences between groups were considered significant at p values < 0.05.

RESULTS

Growth and Feeding Parameters

The CG group showed a significantly higher average weight and weight gain compared to all other groups. There were no significant differences in growth between any of the five groups that received diets containing an FAA mix. The CG group also exhibited a significantly higher feed intake than all other groups. The Def group had a significantly higher feed intake, compared to the FAA50 group, but not significantly different from the other three deficient groups. The significant increase in feed intake in the Def group correlated with a significant decrease in feeding efficiency, compared to the CG group. The (-) Lys group also showed a significantly lower feeding efficiency than the CG group. The feeding efficiency of the FAA50, (-) Met, and (-) Thr groups were not significantly different from any of the other groups. There were no significant differences in survival observed in this study. The results for these parameters are presented in Table 5.5.

Brain Hormone/Gene Expression

There were no significant differences in the gene expression of *orexin*, *CCK*, or *leptin* in the brain between any of the groups analyzed. There was a significant upregulation of *NPY* in the Def group, compared to the FAA50 group. The expression of *NPY* in the (-) Lys, (-) Met, and (-) Thr groups was not significantly different from the FAA50 and Def groups. The results from the expression of hormones in the brain are presented in Figure 5.1.

Gut Hormone/Gene Expression

There were no significant differences observed in the expression of *CCK* or *ghrelin* in the gut, between groups. The expression of *orexin* was significantly higher in the (-) Met group, compared to the FAA50 group, with no other significant differences measured between the rest of the groups. The (-) Thr group exhibited a significant downregulation of *leptin*, compared to the (-) Lys group (Figure 5.2A). The expression of *leptin* in the FAA50, (-) Met, and Def groups was not significantly different from any groups.

The results for the expression of the nutrient-sensing receptors are presented in Figure 5.2B. The expression of *gprc6a* in the (-) Lys group was significantly lower than all groups, except the (-) Thr group. The *gprc6a* expression in the (-) Thr group was also not significantly different compared to the remaining groups. The expression of *casr* was significantly upregulated in all groups except the (-) Thr group, compared to the FAA50 group. No significant differences were observed in the expression of *tas1r2*.

Muscle Free Amino Acid Composition

The (-) Thr group showed a significantly lower level of total FAA in the muscle, compared to the FAA50, (-) Lys, and (-) Met groups, but not significantly different from the CG or Def groups (Figure 5.3). The Def group exhibited a significantly lower level of total IDAA

than all other groups except (-) Thr, and both the CG and (-) Lys groups had levels significantly higher than (-) Thr. The levels of total IDAA in the FAA50 and (-) Met groups were not significantly different from any of the groups except the Def group. Contrary to the IDAA data, the Def group showed the highest postprandial level of DAA, significantly higher than the CG and (-) Thr groups. The (-) Thr group had a significantly lower level of DAA than all groups except CG, and the FAA50, (-) Lys, and (-) Met groups were not significantly different from the CG and Def groups. Significant differences were also observed among the three major limiting amino acids tested in this study. The CG group had a significantly higher level of free lysine than the (-) Thr and Def groups, while the FAA50, (-) Lys, and (-) Met groups were not significantly different from any groups. The level of free methionine was significantly reduced in the Def group compared to all groups except the (-) Met group. No significant differences were observed in the postprandial level of free methionine between any groups, except the Def group. Lastly, both the CG and (-) Lys groups had significantly higher postprandial levels of free threonine than the FAA50, (-) Thr, and Def groups. The level of free threonine was significantly higher in the (-) Met group, compared to the (-) Thr and Def groups.

Among the other analyzed FAA, the postprandial level of free glutamic acid in the muscle was significantly higher in the FAA50 group than the CG and (-) Lys groups. The CG group had a significantly higher level of free asparagine than all other groups, while the Def group had a significantly lower level than the FAA50 and (-) Thr groups. The Def group had significantly a higher level of free glycine, compared to all other groups, and the CG group showed the lowest. The levels of free serine in the (-) Lys and Def groups were significantly higher, compared to the CG, (-) Met, and (-) Thr groups. The FAA50 group showed a significantly higher postprandial level of free glutamine, compared to the (-) Lys group. The

level of free histidine was significantly reduced in the (-) Thr and Def groups, compared to all groups except CG. The CG group exhibited a significantly lower level of free alanine than all other groups, while the Def groups had a significantly increased level compared to the (-) Lys group. The postprandial level of free tyrosine was significantly increased in the Def and (-) Met groups, compared to the CG group. The (-) Lys group had a significantly higher level of free valine than the Def group. The measured level of free phenylalanine in the muscle was significantly higher in the (-) Met group, compared to the CG group. Both the CG and Def groups exhibited lower levels of free isoleucine than the FAA50 group. The level of free proline was significantly reduced in the (-) Thr group, compared to all groups except FAA50. No significant differences were observed in the postprandial levels of free aspartic acid, free arginine, free tryptophan, or free leucine between groups. The results for all postprandial levels of FAA analyzed are presented in Table 5.6 (2 h) and Table 5.7 (24 h).

DISCUSSION

The supplementation of 50% of dietary protein with FAA mix led to significant reductions in growth and feed intake in Zebrafish in this study. In addition to the reduced feed intake, the reduction in growth can be attributed to the high proportion of dietary protein supplied in FAA form, which has also been observed in previous studies that utilized FAA-based diets (Dabrowski et al. 2003, Terjessen et al. 2006; Zhang et al. 2006; Dabrowski et al. 2007; Kwasek et al. 2010). While the overall FAA supplementation decreased growth, the IDAA deficiencies did not have a significant effect on growth compared to the balanced FAA50 diet. With regards to feed intake, there was no significant change in response to the deficiencies of the singular IDAA; however, each of the deficiencies led to numerical increases in feed intake compared to the FAA50 group. Furthermore, the fish significantly increased their feed intake of

the diet that was deficient in all the selected IDAA (lysine, methionine, and threonine), compared to the FAA50 group. This might suggest an apparent additive effect of the dietary IDAA deficiencies. The combined deficiency seemed to be enough to trigger a significant increase in feed intake. This somewhat agrees with the results observed in Dabrowski et al. (2007), where combined deficiencies of lysine, histidine, isoleucine, phenylalanine, and tryptophan in one diet, and arginine, threonine, valine, methionine, and leucine in another diet, increased feed intake in juvenile Midas. The apparent additive effect of the IDAA deficiencies could be due to an increase in the severity of the total IDAA deficiency. The severity of deficiency is characterized by the percentage of the requirement level at which an IDAA is present, with lower percentage levels having a higher severity of deficiency. A study done on rats found that severe deficiency levels (15% and 25% of requirement level) of lysine and threonine individually, induced a significant increase in relative feed intake, compared to the control diet (Moro et al. 2022). However, at more moderate deficiency levels (40% and 60% of requirement level), the relative feed intake was not significantly different from the control diet (Moro et al. 2022). In this study, the deficiency of lysine was 80.6% of the requirement level in the (-) Lys diet, the deficiency of methionine was 83.6% of the requirement level in the (-) Met diet, and the deficiency of threonine was 59.1% of the requirement level in the (-) Thr diet. Based on these results, the singular IDAA deficiencies in this study may not have been severe enough to elicit a significant feed intake response, but the combined deficiency of lysine, methionine, and threonine created a higher severity of deficiency in the Def diet, which may have led to the significant feed intake response in Zebrafish.

Along with a significant increase in feed intake, there was a significant reduction in feeding efficiency observed in the Def group. This helps explain the lack of a significant

decrease in growth in response to the IDAA deficiencies, having consumed more feed to match the growth of the balanced FAA50 group. The singular deficiency of lysine also led to a significant reduction in feeding efficiency in Zebrafish, compared to the CG group. This result seems to be consistent with Yaghoubi et al. (2017), which found that the lysine deficiency increased the feed conversion ratio more than any other IDAA in juvenile Silvery-black Porgy, fed with IDAA deficient diets for 6 weeks.

The significant increase in feed intake in response to IDAA deficiencies in this study is contradictory to previous results observed in fish (Yamamoto et al. 2001; Fortes-Silva et al. 2012). One possible explanation behind this is the model species used. Previous studies were conducted on stomach-possessing species, while this study utilized Zebrafish - a stomachless species. Stomachless fish have been found to utilize diets based on synthetic peptides and FAAs less efficiently than stomach-possessing species (Dabrowski et al. 2003; Zhang et al. 2006). For example, diets based on synthetic FAAs were unable to support growth in Common Carp, but similar FAA-based diets led to positive growth responses in Rainbow Trout (Dabrowski et al. 2003; Zhang et al. 2006). The inability of dietary FAAs to support growth in stomachless species is largely due to an increase in the excretion of dietary FAA from the gills, compared to diets based on complex protein (Murai et al. 1984; Lovell, 1991). These findings help explain the significant reduction in growth observed in the fish that received diets supplemented with FAA in 50%, compared to the CG group. Based on the significant difference in utilization of synthetic FAA-based diets between stomachless and stomach-possessing fish species, it is feasible that the behavioral response to dietary amino acid compositions and deficiencies differs between the two groups as well.

Another plausible explanation behind this discrepancy could lie in the differences in feeding regimes used. In the previous studies, the tested fish were exposed to a diet that contained a balanced IDAA profile and a diet that contained IDAA deficiencies, either simultaneously (Fortes-Silva et al. 2012) or over 8-day intervals (Yamamoto et al. 2001). The fish were able to select between the balanced and deficient diets and reduced their consumption of the deficient diets as a result. In the present study, the fish in each group only received one assigned diet, and the intake of the groups that received deficient diets were compared to those that received the balanced diets, ultimately leading to an increase in the intake of the Def diet. This might suggest that the behavioral response to IDAA deficiencies in fish is different when they are presented with a choice of diets, compared to when they are only exposed to one diet, and that response may change over time. Dabrowski et al. (2007) found that juvenile Midas cichlid initially reduced their feed intake of IDAA deficient diets during the first two days of feeding, however at the conclusion of the 32-day feeding trial, the fish showed a significantly higher feed intake of those same diets, compared to a balanced, FAA-based feed. The initial aversion to the deficient diets supports the results presented in the previous studies (Yamamoto et al. 2001; Fortes-Silva et al. 2012), however it appears that the prolonged exposure to an IDAA-deficient diet triggers an eventual increase in feed intake, compared to balanced diet.

The mechanism behind the detection and response to dietary amino acid deficiencies in form of increased or reduced feed intake lies in the gut-brain axis (Comesaña et al. 2018; Butt and Volkoff, 2019; Blanco et al. 2021; Calo et al. 2021). The gut-brain axis plays a significant role in feed intake regulation and the maintenance of energy homeostasis in animals (Blanco et al. 2021). To facilitate this, receptors found in enteroendocrine cells (EECs) within the digestive tract help detect the presence of micro- and macronutrients. In response to these nutrients, the

EECs then send signal molecules that induce the secretion of enzymes or hormones in the gut, or signals that reach the hypothalamus and facilitate the secretion of hormones (Blanco et al. 2021). This allows for sufficient energy intake to be maintained based on the composition of the diet being consumed. Recent studies have also found that EEC receptors in the digestive tract of fish are responsive to amino acids and affect the expression of appetite-regulating hormones within the gut (Calo et al. 2023).

The role of the gut-brain axis in the feed intake response observed in this study was assessed by measuring the expression of appetite-regulating hormones in the brain and digestive tract, as well as the expression of nutrient signaling genes. The triggering factor behind the increase in feed intake in response to the IDAA deficiencies in this study seems to be the expression of *NPY* in the brain. *NPY* is an orexigenic hormone that stimulates feed intake in fish (Narnaware et al. 2000; Assan et al. 2021). Previous studies have found that the secretion of *NPY* is activated by receptors within the digestive tract that send signals to the brain, and in turn are used to adjust *NPY* expression based on satiety or hunger (Assan et al. 2021). In this study, the expression of *NPY* was significantly upregulated in the Def group, which suggests that the increased release of this hormone may have stimulated the significant increase in feed intake. One study specifically linked a lysine deficiency as a cause for the upregulation of *NPY* in fish (Zou et al. 2022), although that effect was not observed in this study. Within the gut, *orexin* was significantly upregulated in the (-) Met group, compared to the FAA50 group. *Orexin* is an orexigenic hormone that stimulates feed intake in Zebrafish (Novak et al. 2005; Matsuda et al. 2012). The stimulation of *orexin* functions to regulate energy homeostasis (Matsuda et al. 2012) and may have been triggered by the IDAA deficiency. There was a trend in upregulated *orexin* in all groups that received the deficient diets, and the singular deficiency of methionine triggered a

significant upregulation over a balanced FAA-based diet. Additional hormone expression analysis in the gut showed that the (-) Lys group had a significantly higher expression of *leptin* than the (-) Thr group. *Leptin* is an anorexigenic hormone that serves as a satiety signal and reduces feed intake in fish (Blanco and Soengas, 2021). Based on these results it appears that different singular IDAA deficiencies can impact the expression of different hormones within the gut, however neither of these changes in appetite-regulating hormones corresponded directly with a significant difference in feed intake in response to singular lysine, methionine, or threonine deficiencies.

The dietary deficiencies in singular IDAA also induced significant changes in the expression of certain nutrient-sensing receptors. These receptors were recently observed by Calo et al. (2021) to be responsive to dietary amino acids in the gut of Rainbow Trout. In the present study, the expression of *casr* was significantly upregulated in response to deficiencies of lysine, methionine, and the combined deficiency of those two, plus threonine. *Casr* is a calcium-sensing receptor that functions as part of the gut-brain axis, and in mammals, it is the major sensing receptor for aromatic amino acids (Calo et al. 2021). In addition to *casr,* the expression of *gprc6a* was also responsive to the IDAA deficiencies. *Gprc6a* functions as a receptor for small, neutral and basic amino acids, like lysine (Calo et al. 2021). The deficiency of lysine led to a significant downregulation of *gprc6a*, compared to all groups except the (-) Thr group. Contrary to the other two receptors within the gut, the expression of *tas1r2* was not significantly different between any of the groups. *Tas1r2* is a member of the taste receptor family and has been found to be responsive to a wide range of amino acids in Zebrafish (Oike et al. 2007; Calo et al. 2021). The response to many amino acids by *tas1r2* may be a reason why significant differences in expression were not observed in this study, as the diets only significantly differed in levels of the

three selected IDAA. These results support the findings from Calo et al. (2021) that *casr* and *gprc6a* were responsive to IDAA deficiencies and provide evidence that Zebrafish can sense amino acid deficiencies within the gut, and these receptors could play a role in behavioral responses to IDAA deficiencies. As neither of the receptors that showed significant up-/downregulations in Zebrafish correlated with the appetite-regulating hormones, their role in the change in feeding response needs to be further evaluated.

An interesting result observed in this study was that the group that received the diet that was deficient in all three of the major limiting amino acids presented the highest postprandial level of DAA. In contrast, this group also showed the lowest postprandial level of IDAA. This significant imbalance between IDAA and DAA in the muscle FAA pool highlights a negative effect of dietary IDAA deficiencies. As mentioned previously, in the presence of dietary IDAA deficiencies, especially the major limiting amino acids, there is a reduction in protein synthesis and a reduction in nitrogen retention (Ballantyne, 2001). The breakdown of body proteins and synthesis of new protein from the FAA pool is a constant process that occurs in fish(protein turnover). With the deficiency of IDAA in the FAA pool from the diet, the levels of protein synthesis and protein degradation can become imbalanced, resulting in an excess of DAA from degraded body proteins. A previous study found that in fish fed a diet containing a low IDAA/DAA ratio, the muscle FAA pool exhibited a significantly lower IDAA/DAA ratio as well, compared to the balanced diets (Gómez-Requeni et al. 2003). The feeding of this deficient diet also significantly reduced the nitrogen retention in the studied fish (Gómez-Requeni et al. 2003). While there was an assumed increase in protein breakdown in the fish fed with the IDAA deficient diet in this study, there was not a significant reduction in growth. This result was also observed by Gómez-Requeni et al. (2003) and was attributed to a numerical increase in feed

intake, and a significant increase in feed conversion ratio, similar to the results observed in this study.

The feed intake response observed in this study is directly opposite to those observed in mammals (Gloaguen et al. 2012; Koehnle et al. 2003). A possible explanation for the difference in feeding response to IDAA deficiencies between mammals and fish is the difference in energy costs associated with the excretion of nitrogenous wastes (Dabrowski et al. 2007). In mammals, excess amino acids are catabolized to produce ammonia, which is further converted into urea for excretion (Wright, 1995). This process requires high energy input, making it severely inefficient for mammals to consume diets that promote excessive excretion of nitrogenous waste. In fish however, the process requires much less energy, as a high proportion of nitrogenous waste is simply excreted to the water as ammonia. Additionally, fish are more tolerant of higher ammonia levels in the blood than mammals (Dabrowski et al. 2007). With the lower energy cost of excreting excess nitrogen, fish are better able to increase feed intake of deficient diets to meet their amino acid requirements, compared to mammals.

Overall, the results from this study showed that although the singular deficiency of the three main limiting amino acids did not induce significant changes in feed intake, the combined deficiency of the three IDAA (lysine, methionine, and threonine) significantly increased the feed intake of juvenile Zebrafish. This increased feed intake prevented the IDAA deficiencies from significantly reducing growth, however as a result, the feeding efficiency of the Def diet was reduced. The reduced feeding efficiency could be due to a decrease in protein synthesis as a result of an imbalanced FAA pool, signified by a significantly lower postprandial level of IDAA in the muscle FAA pool, but a significantly higher level of DAA. The reduction in feeding efficiency could also be due to an increase in the excretion of dietary FAA, which has been

observed in other stomachless fish species. The analysis of appetite-regulating hormones suggests that an upregulation of orexigenic hormone *NPY* might play a role behind the increased feed intake in response to the Def diet. Further research is required to determine the direct pathway behind the upregulation of *NPY*, or to identify other receptors or hormones involved in the response to dietary IDAA deficiencies. The results from this study in conjunction with other similar studies suggests that the behavioral response in fish may be different between stomachless and stomach-possessing species, when exposed to balanced and deficient diets vs. prolonged exposure to deficient diets alone, increasing their feed intake of deficient diets when it is the only food source.

CHAPTER 6

THE POSTPRANDIAL FREE AMINO ACID DYNAMICS OF THREE FISH SPECIES FED WITH A CASEIN-GELATIN DIET VS. A FREE AMINO ACID-SUPPLEMENTED DIET

ABSTRACT

Analysis of the postprandial free amino acid (FAA) pools has been used as a means of investigating dietary protein utilization in fish. However, many other environmental, nutritional, and physiological variables can impact these FAA pools, which limits the ability to draw conclusions from different studies and different species. The objective of this study was to track the postprandial FAA levels in the plasma, liver, and muscle of fish fed with an intact protein or FAA-based diet, in three species; 1) Largemouth Bass (LMB) (*Micropterus nigricans*) – warm-water, stomach-possessing carnivorous species; 2) Walleye (*Sander vitreus*) – cool-water, stomach-possessing carnivorous species; and 3) Zebrafish (*Danio rerio*) – tropical, stomachless omnivorous species. Two diets were formulated for this study, a diet based on intact casein and gelatin (CG), and a diet with 50% of its protein supplied in FAA form (FAA50). Forty-two fish from each species were utilized, with one group of 21 receiving the CG diet, and the other 21 receiving the FAA50 diet. All fish were starved for 24 hours prior to the final feeding before sampling. Both LMB and Walleye were administered their respective diets by force-feeding. The Zebrafish were randomly distributed into 2 (10 L) tanks (one tank per dietary treatment) and received their respective diets for 6 days prior to the 24 h starvation period before sampling. On the day of sampling, the respective diets were added to the surface of the tanks, and the feeding behavior was carefully observed to ensure all sampled fish consumed the experimental diet. Three fish were sampled at each time point, with three samples (plasma, liver, and muscle) taken from each fish. The plasma was sampled from the caudal vein. Samples were taken prior to

feeding (0 h) and then at 0.5, 1, 2, 3, 6, and 12 h after feeding, for all species. There was a significant three-way interaction between the diet, species, and postprandial time on the total FAA, indispensable amino acids (IDAA), and dispensable amino acid levels in the plasma, liver, and muscle. This indicates that the postprandial FAA patterns were significantly different between species and in response to the different diets. Comparisons between the postprandial FAA dynamics of LMB, Walleye, and Zebrafish showed that in stomach-possessing species, the FAA50 diet led to a more rapid absorption of dietary amino acids than the CG diet. This rapid absorption ultimately led to fewer correlations between the dietary IDAA profile and the IDAA levels in the postprandial FAA pools. This suggests that the rapid absorption of dietary FAA was less balanced than that of intact protein. The absorption of dietary FAA in the cool-water Walleye seemed to be slower and more balanced after feeding than the warm-water LMB. This was supported by more significant correlations with the dietary IDAA profile of the FAA pools in the Walleye, and more sustained peaks than those seen in the LMB. In both LMB and Walleye, the peak FAA levels were similar between the two diets. Markedly different results were seen in the stomachless Zebrafish. The postprandial peaks of FAA typically occurred at the same time in Zebrafish fed with the CG or FAA50 diet. Additionally, the levels of FAA were noticeably lower after feeding with the FAA50 diet. Overall, the results from this study provide a reference for temporal differences in FAA dynamics between species with different physiological characteristics, when fed diets with intact protein or supplemented with free amino acids.

INTRODUCTION

The free amino acid (FAA) pool is the mixture of individual, unbound amino acids that are available within the fish's body for catabolic and anabolic processes. Analyzing the

postprandial FAA pools, mainly in the blood plasma, liver, and muscle, has been commonly used to assess the availability of dietary amino acids (Murai et al 1987; Rønnestad et al. 2000; Zhang et al. 2006; Kwasek et al. 2009; Ambardekar et al. 2009; da Cruz et al. 2021). This analysis provides a way to track the dietary amino acids as they are absorbed and utilized throughout the body (Carter et al. 2001; Dabrowski et al. 2003; Kwasek et al. 2009).

The quality (Kaushik and Luquet, 1980; Mente et al. 2003), digestibility (Yamamoto et al. 1998; Larsen et al. 2012), and molecular form of dietary protein have been found to have a significant impact on the postprandial FAA pools (Zhang et al. 2006; Ambardekar et al. 2009; Kwasek et al. 2009; Kwasek et al. 2010). With relation to protein digestibility, Yamamoto et al. (1998) and Larsen et al. (2012) found that diets based on soybean meal caused a delay in FAA peaks in the plasma compared to a fishmeal-based diet, likely related to the decreased digestion efficiency of plant protein in fish. In response to differences in molecular form of dietary protein, studies have found that diets formulated using FAA resulted in a significantly earlier peak of amino acids in the plasma compared to intact protein diets, but also returned to fasting levels much quicker (Yamada et al. 1981; Ng et al. 1996; Ambardekar et al. 2009). These studies provide support for the use of postprandial FAA pools as a means of investigating dietary protein utilization in fish; however, other factors besides the dietary protein can have a significant impact on the FAA dynamics within the body and how those can be compared among different studies.

Water temperature plays a role in the postprandial dynamics of the FAA pools (Smith, 1989; Ng et al. 1996; Conceição et al. 1998; Zhang et al. 2006). Specifically, FAA absorption and evacuation rates have been reported to increase as water temperatures increase (Conceição et al. 1998). This has led to earlier observed peaks of FAA levels in species with higher rearing

temperatures compared to those at lower temperatures (Murai et al. 1987; Ogata, 1986). The digestive physiology of fish has also been found to have a significant impact on the postprandial FAA pool (Dabrowski and Portella, 2005; Zhang et al. 2006). In stomachless species, dietary amino acids or peptides led to a decrease in the postprandial concentration of indispensable amino acids (IDAA; Zhang et al. 2006). In contrast, the inclusion of FAA in a diet for Rainbow Trout (*Oncorhynchus mykiss*) a stomach-possessing species, led to a significant increase in the concentration of those FAA in the plasma (Tantikitti and March, 1995).

Due to the large number of environmental, nutritional, and physiological variables that can impact the postprandial FAA pool, the ability to compare results from different studies and different species can be limited. Differences in holding temperatures (Costas et al. 2012), digestive tract morphology (Dabrowski and Portella, 2005; Zhang et al. 2006; Cowey, 2013), diet formulations (Yamamoto et al. 2005), diet digestibility (Yamamoto et al. 1998; Larsen et al. 2012), etc. can all affect absorption rates and FAA concentrations in the postprandial levels of FAA in different tissues. Therefore, a direct comparison of the postprandial FAA dynamics between different species under controlled conditions with controlled diets is imperative. The objective of this study was to determine the postprandial FAA levels in the blood plasma, liver, and muscle of fish fed with an intact protein-based diet, and a diet containing FAA as half of its protein source in the following three species;

I. Largemouth Bass (LMB) (*Micropterus nigricans*) – warm-water, stomach-possessing carnivorous species
II. Walleye (*Sander vitreus*) – cool-water, stomach-possessing carnivorous species
III. Zebrafish (*Danio rerio*) – tropical, stomachless omnivorous species.

MATERIALS AND METHODS

Experimental Conditions

All trials were conducted at the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University-Carbondale (SIUC), IL. The experiment was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of SIUC. The SIUC Institutional Animal Care and Use approved all of the protocols performed (protocol #21-006).

For LMB and Walleye, the experiment was carried out using a semi-recirculated aquaculture system, with groundwater as the water source. The system was equipped with two mechanical (sand) filters (Pentair, Minneapolis, MN) and a bio-filter. The system consisted of 30 (280 L) black tanks. The temperature was maintained at 21°C for Walleye and 24°C for LMB with a pH of 7.45. The water flow into each tank was kept at 200 ml/min.

For Zebrafish, the experiment was also carried out using a semi-recirculated aquaculture system (Pentair Aquatic Eco-systems, Cary, NC). The system was equipped with two mechanical filters, a carbon filter and UV light. The temperature was kept at 27°C, with a pH of 7.33. The water flow was set at 100 ml/min in each tank.

Diet Formulation and Treatment Groups

Two semi-purified diets were formulated for this study (Table 6.1). The CG diet was supplied with 100% of its protein in the form of purified casein and gelatin. The FAA50 diet contained 50% of its protein in purified intact form (casein and gelatin) and the other 50% supplied in crystalline amino acid form. The composition of the FAA mix is provided in Table 6.2. The diets utilized in this study were based on previous reports (Dabrowski et al. 2003; Kwasek et al. 2010) for salmonids and cyprinids, respectively, and were formulated to be

isonitrogenous and isolipidic and meet the essential nutrient requirements for each of the three species utilized (NRC, 2011). The analyzed composition of the formulated diets is presented in Table 6.3. Six groups of fish were utilized in this study, LMB fed with the CG (LMG-CG) or FAA50 diet (LMB-FAA50, Walleye fed with the CG (W-CG) or FAA50 diet (W-FAA50), and Zebrafish fed with the CG (ZF-CG) or FAA50 diet (ZF-FAA50).

Diet Administration

Largemouth Bass and Walleye

Due to low feed intake of purified diets and to avoid any potential leaching, the juvenile LMB and Walleye were administered their respective diets through force-feeding. Forty-two LMB (106.43 ± 30.63 g) and forty-two Walleye (65.64 ± 14.26 g) were used. The fish were anesthetized using MS-222 (0.10 mg/mL) prior to force-feeding to minimize stress (Topic Popovic et al. 2012). The diets were powderized and mixed with water (1:1.5) to make the solution appropriate for feeding. Each fish was weighed prior to feeding, and they were fed a meal equivalent to 1% of their body weight, representing one meal of a standard daily feeding rate of 3% body weight. The feed was administered into the esophagus through a syringe (60 mL) equipped with a catheter (5 in long, 4.7 mm diameter). The catheter was carefully inserted into the fish's mouth and esophagus until a resistance was felt. When the resistance was reached, the catheter was slightly pulled back and the feed was administered to prevent forceful contact to the esophagus. Once the fish had been fed, it was released into the holding tank (280 L black tank) until sampling. The fish in the holding tank were observed consistently to ensure proper recovery from the anesthesia and complete ingestion of the feed. Fish that experienced regurgitation were replaced, and the force-feeding was conducted on a new fish.

Zebrafish

Forty-two adult Zebrafish (0.94 ± 0.10 g) were randomly distributed into 2 (10 L) tanks (one tank per dietary treatment). The Zebrafish received their respective diets (~0.8 mm) for 6 days, and then feed was withheld for 24 h prior to the final feeding for sampling. On the day of sampling, the Zebrafish received one meal (1% total tank biomass) with their respective diets and were then sampled at the predetermined time points. The feeding was observed to ensure all sampled fish consumed the experimental diet. All fish exhibited feeding behavior at the top of the water column when the diets were added, and the feeds were readily consumed.

Sampling

Largemouth Bass and Walleye

All LMB and Walleye were starved for 24 hours prior to sampling. Samples were taken prior to feeding (0 h) and then at 0.5, 1, 2, 3, 6, and 12 h after feeding. The 0 h sampling represents 24 h starved fish. Three fish were sampled at each time point, with three samples (plasma, liver, and muscle) taken from each fish. To collect the blood samples, fish were anesthetized with MS-222 (0.10 mg/mL). The blood was collected from the caudal vein using a heparinized (3 mg mL $NaCl^{-1}$) needle (22 gauge). The blood samples were stored on ice and quickly centrifuged (4°C, 2000 xG for 10 min) to obtain the plasma. Once the blood was collected, the fish were euthanized with an overdose of MS-222 to collect the remaining samples. The muscle samples were collected from the white muscle in the dorsal section of the fish. The liver samples were collected after the muscle samples. All samples were immediately stored at -80°C after collection for FAA analysis.

Zebrafish

All Zebrafish were starved for 24 hours prior to sampling. Samples were taken prior to feeding (0 h) and then at 0.5, 1, 2, 3, 6, and 12 h after feeding, the same timepoints as the LMB and Walleye. Three fish were sampled at each time point, with three samples (plasma, liver, and muscle) taken from each fish. Prior to sampling, the fish were anesthetized in a water bath containing 0.10 mg/mL MS-222. For blood collection, a shallow diagonal incision was made at the base of the caudal peduncle, severing the caudal vein. Once the incision was made, a heparinized (3 mg mL $NaCl^{-1}$) pipette tip was used to collect the expelled blood. After blood collection, the fish were euthanized with an overdose of MS-222. After euthanizing, the liver and muscle were harvested from the fish and stored at -80^{o}C for FAA analysis.

Statistical Analysis

The postprandial FAA levels were analyzed with a three-way ANOVA, to evaluate interaction effects of the species, diet, and postprandial time on the FAA dynamics in the blood plasma, liver, and muscle. Before the ANOVA, Shapiro–Wilks' test was performed to assess the normality distribution, and homoscedasticity was assessed by Levene's test. Interactions were considered significant at $p < 0.05$. Pearson correlation coefficients between the dietary IDAA profile and the IDAA patterns within the sampled FAA pools were calculated, and the correlations were considered significant at $p < 0.05$.

RESULTS

Postprandial FAA Dynamics

The results from the three-way ANOVA showed there was a significant three-way interaction between the diet, species, and postprandial time on the TFAA levels in the plasma ($p < 0.001$), liver ($p < 0.001$), and muscle ($p = 0.026$). The same significant three-way interaction

was observed on the IDAA levels in the plasma ($p < 0.001$), liver ($p < 0.001$), and muscle ($p = 0.002$), and on the DAA levels in the plasma ($p < 0.001$), liver ($p < 0.001$), and muscle ($p = 0.040$). The postprandial dynamics in each FAA pool of each species are presented in Figure 6.1 (TFAA), Figure 6.2 (IDAA), and Figure 6.3 (DAA).

Largemouth Bass

The TFAA and DAA levels in the plasma within each group of LMB showed similar postprandial patterns. Both the TFAA and DAA levels in the plasma showed sharp declines at 0.5 h after feeding with the CG diet, but not the FAA50 diet. The TFAA and DAA in the LMB-FAA50 group showed an earlier peak at 2 h, but the LMB-CG group had a more sustained peak from 3 – 6 h and stayed at higher levels than the LMB-FAA50 group through the 12 h sampling. The same peak at 2 h occurred in the IDAA levels in the plasma of the LMB-FAA50 group. A peak in IDAA levels was observed at 3 h in the LMB-CG plasma. This IDAA peak in the plasma of the LMB-CG group was lower than that of the LMB-FAA50 group, although only a gradual decrease was observed after the peak and the IDAA levels were consistently higher than the LMB-FAA50 group 3 – 12 h.

The TFAA and IDAA levels in the liver of LMB stayed relatively stable, the only noticeable differences were a slightly higher TFAA level in the LMB-CG group 1 h after feeding, and a small peak in IDAA in the LMB-FAA50 group at 2 h. The DAA levels in the LMB-FAA50 liver decreased from 0 to 1 h, and then sharply increased at 2 h, and maintained similar DAA levels to the LMB-CG group for the rest of the sampling times.

The LMB-FAA50 group experienced a significant peak in TFAA at 6 h in the muscle. The LMB-CG group showed a similar peak in the muscle TFAA at 6 h, however this peak was much lower than that of the LMB-FAA50 group. This 6 h peak in the muscle was also observed

in the IDAA and DAA levels in the LMB-FAA50 group, and in the DAA levels of the LMB-CG group. There was a decrease in IDAA levels from 0 to 1 h in the LMB-CG muscle, followed by a plateau back to levels near those in this LMB-FAA50 group. There was also an immediate decrease in TFAA and DAA levels in the muscle following feeding with the CG diet in LMB from 0 – 0.5 h.

Walleye

Both groups of Walleye showed two peaks in the plasma TFAA and IDAA levels. The W-CG group showed peaks in plasma TFAA and IDAA at 1 and 12 h after feeding. The TFAA and IDAA in the plasma peaked at 0.5 h, and then at 2 – 3 h in the W-FAA50 group. Slightly different results were observed in the plasma DAA levels of these two groups. The same peak at 2 – 3 h was observed in the DAA levels of the W-FAA50 group, however the peaks in the W-CG group were not seen and the DAA levels in the W-CG plasma were lower than those in the W-FAA50 group at all time points, except for 1 and 12 h.

The W-FAA50 group showed a significant peak in TFAA and DAA at 1 h in the liver, followed by a steady decrease and level off observed at 6 h. The TFAA and DAA levels in the W-CG liver did not show any peaks and were consistently lower than those of the W-FAA50 group. The IDAA levels in the W-FAA50 showed the same 1 h peak in the liver, however the levels of IDAA dropped down to similar levels at the W-CG group at 6 h and did not stay at higher levels, as was observed in the TFAA and DAA levels.

The Walleye muscle did not show any significant peaks in TFAA levels, but the levels in the W-FAA50 muscle were numerically higher than the W-CG muscle at all sampling timepoints 3 – 12 h. Slight peaks were observed in IDAA levels at 12 h in both Walleye groups, with the W-CG peak numerically higher than that in the W-FAA50 muscle. There was a decrease in muscle

DAA immediately after feeding in the W-FAA50 group, then the DAA levels in the muscle remained steady in both groups until 6 h, where sharp decreases were observed.

Zebrafish

The plasma of both the ZF-CG and ZF-FAA50 groups followed the same postprandial pattern of TFAA levels. There was a sharp decrease in TFAA immediately after feeding at 0.5 h, followed by a peak in plasma TFAA at 6 h, and slight decreases occurring at 12 h. While the pattern was the same, the ZF-CG plasma showed markedly higher TFAA levels than the ZF-FAA50 group at each point after 2 h. This same trend was observed in the plasma IDAA levels between these two groups. The same relative pattern was also observed in the plasma DAA levels of the ZF-CG and ZF-FAA50 groups, however the peaks of DAA were muted and not as drastic compared to those observed in the TFAA and IDAA levels.

The Zebrafish liver was less responsive, no strong peaks in TFAA, IDAA, or DAA were observed in response to either of the diets. The levels of TFAA and DAA in the liver of the FAA50 fed Zebrafish rose above those of the ZF-CG group from 0.5 – 1 h, and then were numerically lower 3 – 12 h. The levels of IDAA in the liver of the FAA50 fed Zebrafish were at or above the levels seen in the CG fed Zebrafish at all postprandial timepoints.

The muscle of the ZF-CG group stayed stable through the sampling timepoints, showing only slight changes TFAA and IDAA levels over time. The Zebrafish muscle was a bit more responsive to the FAA50 diet, with a peak in TFAA occurring at 2 h, followed by a sharp decrease at 3 h, and slight increase from 3 – 12 h. The same 2 h peak and 3 h decline in the ZF-FAA50 muscle was observed for the IDAA levels, and for all timepoints except the 2 h peak, the muscle IDAA levels were lower than the ZF-CG group. The levels of DAA in the muscle were

similar between the ZF-FAA50 and ZF-CG groups, with the levels being slightly higher in the ZF-FAA50 group at a 2 h peak, and then slightly lower at a 3 h decline.

Dietary and FAA Pool IDAA Correlations

The most significant correlations with the dietary IDAA profile were observed in the plasma FAA pool (Table 6.4). The FAA50 diet led to a significant correlation 2 h after feeding in the LMB, where the CG diet led to a more sustained correlation, with significant correlations observed at 2 and 3 h in the LMB-CG group. The Walleye showed more significant correlations with the dietary IDAA profile in the plasma than either the LMB or Zebrafish. The W-CG group had significant correlations at all time points except at 0 h, and the W-FAA50 group had significant correlations with the dietary IDAA profile at each postprandial time 1 – 12 h. In Zebrafish, the only significant correlation with the dietary IDAA profile was at 0.5 h in the ZF-CG group in the blood plasma.

In the liver, only the FAA50 fed Walleye showed any significant correlations with the dietary IDAA profile, with a significant correlation occurring at 1 h, and then dropping back to nonsignificant levels (Table 6.5). None of the other groups showed a significant correlation of the liver IDAA pattern with the dietary IDAA profile, at any of the postprandial timepoints.

The free IDAA pattern in the muscle of all fish at each postprandial timepoint was negatively correlated with the dietary IDAA pattern, but none of these correlations were significant (Table 6.6).

DISCUSSION

This study aimed to identify differences in the temporal dynamics of dietary amino acid distribution through the FAA pools within the body of three different species, in response to diets with intact protein, or supplemented with crystalline amino acids. The total FAA levels observed

in this study ranged from 1.65 – 7.68 μmol/ml in the blood plasma, 1.30 – 4.37 μmol/g in the liver, and 29.92 – 67.25 μmol/g in the muscle. These levels are comparable with previous studies that assessed postprandial FAA levels (Yamada et al. 1981; Ogata, 1986; Espe et al. 1993; Yamamoto et al. 1998; Sunde et al. 2004; Rungruangsak-Torrissen et al. 2009). There was a significant three-way interaction between species, diet, and time on the TFAA, IDAA, and DAA levels in all three FAA pools, which indicates the temporal FAA dynamics were significantly different between all groups. Further context into both the dietary contribution and relative balance of dietary IDAA absorption to the postprandial FAA pools in this study was provided by assessing correlations between dietary IDAA compositions and the IDAA profiles of the tissue FAA pools, as was done in previous studies (Ogata, 1986; Lyndon et al. 1993; Ng et al. 1996; Carter et al. 2000; Mente et al. 2021). High correlations with the dietary IDAA profile suggest that the IDAA within the FAA pool is proportional to that in the diet and points to a more balanced absorption of dietary protein, whereas a low correlation points to an unbalanced absorption as the IDAA were absorbed disproportionately to the levels of IDAA supplied in the diet (Plakas et al. 1980; Ng et al. 1996). The three FAA pools analyzed in this study in blood plasma, liver, and muscle, represent important stages of amino acid metabolism, distribution, and utilization.

Blood Plasma

The blood plasma represents the transportation system for FAA within the body. The plasma transports intestinally absorbed FAA to the liver, and then transports those dietary amino acids and DAA synthesized in the liver to other organs and tissues. The plasma samples in this study were taken from the caudal vein, similar to other studies that evaluated postprandial FAA dynamics in response to different dietary proteins (Murai et al. 1987; Espe et al. 1993; Ng et al.

1996; Yamamoto et al. 1998; Larsen et al. 2012; Brezas and Hardy, 2020). The FAA pools within the plasma were highly responsive to feeding, and the temporal patterns of TFAA, IDAA, and DAA concentrations were significantly different between diets and species. In LMB, the levels of TFAA, IDAA, and DAA in the plasma peaked 2 hours after feeding with the FAA50 diet. The plasma IDAA composition at this peak was significantly correlated with the dietary IDAA profile, suggesting this peak reflected an influx of successfully absorbed IDAA from the diet. The peak was short however, as immediately after these peaks, there was a sharp decline at 3 h. This sharp, dramatic peak possibly signifies a rapid influx and then depletion of dietary FAA in the plasma absorbed, as the rapid absorption leads to an excess of amino acids that cannot be utilized or stored and are therefore excreted (Karlsson et al. 2006). Feeding LMB with the CG diet led to delayed, but more sustained peaks. The delay in the peak of FAA levels after feeding with the CG diet is due to the slower digestion and subsequent absorption of amino acids from intact protein, compared to dietary FAA (Ambardekar et al. 2009). The peak in TFAA and DAA occurred at 3 h in the LMB-CG group, and lasted through 6 hours after feeding, before falling back to initial levels at 12 h. The plasma IDAA levels also peaked at 3 h following feeding with the CG diet in LMB, with only a gradual decrease in IDAA at 6 and 12 h. This peak in IDAA was lower than the peak observed after feeding with the FAA50 diet. The plasma FAA pool of the CG fed LMB also showed more correlations with the dietary IDAA composition, with significant correlations occurring as the IDAA levels were increasing at 2 h, and first peaking 3 hours after feeding. The peaks of FAA in the plasma in response to the CG diet are consistent with the timing of peaks seen in other species reared at a similar water temperature, including Nile Tilapia (*Oreochromis niloticus*) reared at 25 ^{o}C (Yamada et al. 1982), and Channel Catfish (*Ictalurus punctatus*) (Ambardekar et al. 2009) reared at 24 – 25 ^{o}C. Both species also showed

earlier peaks in plasma FAA when fed diets with supplemented FAA (Yamada et al. 1982; Ambardekar et al. 2009), similar to the results in this study.

The FAA levels in the plasma of Walleye showed different temporal patterns compared to the LMB. Previous studies have typically seen slower peaks in plasma FAA levels in cold-water species, due to slower metabolism and a longer time to complete digestion and nutrient absorption (Yamada et al. 1981; Murai et al. 1987; Smith, 1989; Carter et al. 2001). The highest peaks of TFAA and IDAA in the plasma were observed at 2 and 3 h in Walleye fed with the FAA50 diet. The 2 h peak was the same time the peak was observed in the LMB fed with the FAA50 diet; however, the peak was more sustained in the Walleye. The highest peak of plasma TFAA and IDAA in the Walleye fed the CG diet occurred at 12 h after feeding. As there were no additional time points analyzed between the 12 h and the baseline (0 h) sample, it is unknown whether this peak was sustained as seen in the W-FAA50 group. Previous studies on cold-water fish fed with intact protein have shown peak FAA levels past this 12 h post-feeding point (Yamada et al. 1981; Murai et al. 1987), and also shown similar FAA patterns in the plasma when fed with intact protein and diets with low molecular weight proteins (Murai et al. 1987; Espe et al. 1993).Therefore, it is logical to assume the 12 h FAA peak observed in the plasma of the CG fed Walleye was sustained past this 12 h point, similar to the sustained peak in the W-FAA50 group. In comparison to the LMB, the Walleye also showed more significant correlations between the plasma and dietary IDAA levels over time. Significant correlations were observed at all time points starting with 0.5 h in the CG fed Walleye, and all time points after 1 h in the FAA50 fed Walleye. The sustained correlations possibly suggest that the absorption of dietary IDAA into the plasma was more balanced as it was proportional to the IDAA profile of the diet for a longer period of time. This has also been observed in cold-water species Atlantic cod

(*Gadus morhua*) (Lyndon et al. 1993) and is theorized to be a result of slower metabolism associated with cold-water species.

Interestingly, there were also peaks in TFAA and IDAA in the plasma at 0.5 and 1 h in the W-FAA50 and W-CG groups, respectively. These peaks were slightly lower than the later peaks, and were shorter-lived, with decreases in levels observed at the next sampling time after the first peak in each group. While both sets of peaks followed the same trend as observed in the LMB where the FAA50 diet led to earlier peaks, the peaks at 0.5 h and 1 h in the W-FAA50 and W-CG groups respectively, were earlier than those in the warm-water LMB fed the same diets. Given the other results that represent the slower metabolism of Walleye in this study compared to LMB, including the more sustained correlations with the dietary IDAA in both the W-CG and W-FAA50 groups, the peaks in plasma FAA at 0.5 h and 1 h, present a contrast. This contrasting result could be due to a difference in stress responses to handling between the species. While the fish were anesthetized prior to force-feeding and sampling as a means of reducing stress, this does not fully eliminate stress responses in fish (Topic Popovic et al. 2012). Stress responses to handling have been shown to induce strong metabolic reactions (Morales et al. 1990; Aragao et al. 2008; Costas et al. 2011). This metabolic response can be rapid (López-Patiño et al. 2014) and has led to dramatic changes in plasma FAA levels (Morales et al. 1990; Vijayan et al. 1997; Costas et al. 2011). Typically, the most immediate change in the plasma FAA levels is a temporary peak in FAA following a stress-inducing event (Morales et al. 1990; Vijayan et al. 1997). In Rainbow Trout, the plasma FAA levels peaked 2 h after handling, followed by a significant reduction at 4 h, and a return to initial FAA levels at 6 h (Morales et al. 1990). The increase in plasma FAA has been associated with the release and catabolism of DAA to provide energy during the stress response in fish (Costas et al. 2011). These studies did not evaluate

stress after handling for feeding purposes, so any effect on the metabolism of dietary FAA was not assessed. It is feasible that a stress-induced metabolic response after force-feeding could have led to a rapid, temporary influx of dietary IDAA into the plasma, exhibited by the peaks at 0.5 and 1 h in the W-FAA50 and W-CG groups, respectively. Although the LMB were also force-fed in this study, the metabolic response to stress is species dependent (Aragao et al. 2008), so it is possible that the Walleye exhibited a more drastic change in metabolism after handling and force-feeding than the LMB.

While the postprandial FAA patterns were a bit different between LMB and Walleye, both diets led to peaks of TFAA, IDAA, and DAA in the plasma, suggesting both species were able to sufficiently absorb amino acids from both forms of dietary protein. In Zebrafish, a different pattern was observed. It is worth noting that the Zebrafish were not force-fed like the LMB and Walleye, so it is feasible that some of these differences could stem from differences in feeding methods. However, based solely on the differences in physiological characteristics, its lack of stomach provides perhaps the most important difference as it pertains to dietary protein utilization, compared to LMB and Walleye. Differences have been found in the ability of stomach-possessing and stomachless fish to utilize dietary proteins, with stomachless fish typically less efficient at absorbing and utilizing dietary low molecular weight protein for growth (Kaushik and Dabrowski, 1983; Dabrowski et al. 2003; Terjessen et al. 2006; Zhang et al. 2006). This difference between species has been attributed to increased excretion of low molecular weight dietary protein through the gills in stomachless species (Murai, 1984; Zhang et al. 2006; Dabrowski et al. 2003). Evidence of this can be seen in the plasma FAA pools of the Zebrafish in this study. While both the CG and FAA50 diets led to sharp declines in plasma TFAA and IDAA immediately after feeding, in the CG fed Zebrafish, there was a gradual increase after this

decline, and a peak of TFAA and IDAA in the plasma at 6 h. In the FAA50 fed Zebrafish, the TFAA and IDAA levels stayed relatively constant after this decline, with a small peak in IDAA at 6 h, the same time point as in the CG fed Zebrafish. However, this peak was much lower than the CG fed fish, and the levels of TFAA, IDAA, and DAA in the plasma were markedly lower at all time points past 1 h in the FAA50 fed Zebrafish. Additionally, Zebrafish was the only species in which the plasma IDAA profile was never significantly correlated with the dietary IDAA profile after feeding with the FAA50 diet, which suggests the absorption of the dietary FAA was inefficient and never proportional to the IDAA composition provided in the diet. This provides further evidence that the absorption of dietary amino acids from FAA-based diets is reduced in stomachless fish, as the plasma IDAA composition in the ZF-FAA50 group never reflected that of the diet.

Liver

While the temporal differences were not as dynamic as those seen in the plasma, the postprandial FAA dynamics in the liver were still significantly different as result of the diet, species, and time interaction. The liver serves as the control center for the amino acid metabolism and its FAA pool consists of not only imported dietary amino acids from the intestine, but also DAA synthesized *in vivo* and exported DAA from degraded body proteins. As a result, the liver FAA pool seems to be less prone to sudden changes in FAA levels after feeding. Consequently, there were fewer significant correlations with the dietary IDAA profile than those observed in the plasma FAA pools.

In both the LMB and Zebrafish, the fish fed with the CG diet showed liver FAA concentrations that were fairly level across the sampling timepoints. The fish fed with the FAA50 diets in these species were a bit more responsive. The levels of TFAA, IDAA, and DAA

in the liver of the FAA50 fed LMB showed gradual decreases over the first hour after feeding, slight peaks at 2 h, and then a level off at concentrations similar to the CG fed LMB. The Zebrafish fed with the FAA50 diet showed increases in TFAA, IDAA, and DAA in the liver immediately after feeding, and then gradual decreases to levels at or slightly below those in the ZF-CG group, 2 hours after feeding. Neither of the liver IDAA concentrations in these species showed any significant correlations with the dietary IDAA profiles.

In contrast to LMB and Zebrafish, the FAA profile over time in the liver of Walleye was noticeably different between diets. Walleye was the only species to show a dramatic peak in liver IDAA, and it only occurred after feeding with the FAA50 diet. This peak occurred at 1 h post-feeding and was the only point where any of the liver IDAA profiles among any groups showed a significant correlation with the dietary IDAA profile. The peak in liver IDAA occurred between the first plasma IDAA peak at 0.5 h in the Walleye-FAA50 group, and the second peak at 2 h. Considering no other species showed a significant peak or correlation with the dietary IDAA profile in the liver prior to peaks in the plasma, this result may have been affected by the potential stress response in the Walleye force-fed with the FAA50 diet. However, the same response was not observed in the liver of the W-CG group, and without analysis of stress-related markers, any potential stress responses in the study are purely speculative.

Muscle

The muscle FAA pool is the last in the sequence of the three FAA pools sampled in this study as it pertains to the appearance of postprandial FAA peaks (Carter et al. 2000). Free amino acids within the muscle consist of those absorbed from the diet or synthesized in the liver that are available for tissue synthesis, and DAA from degraded muscle proteins as a result of protein turnover. As a result of its reliance on the active transport of dietary and synthesized FAA from

the liver through the plasma, the FAA pool in the muscle seems more resistant to sudden changes after feeding, similar to the liver. However, measured postprandial changes still occur, and the significant interaction between the diet, species, and time on the levels of TFAA, IDAA, and DAA in the muscle indicates that the postprandial FAA dynamics in the muscle were significantly different between the six groups.

The muscle FAA pool within the LMB was the most responsive after feeding among the three species tested in this study. Although the peaks of FAA in the plasma occurred at different times in response to the two diets, the peaks within the muscle occurred at the same time. The TFAA and DAA peaked at 6 h in the muscle of LMB, but the levels were markedly lower in the CG fed LMB. Additionally, only the FAA50 fed LMB showed a peak in IDAA within the 12 h postprandial sampling time, occurring at 6 h and suggesting that the supply of IDAA was either reduced or delayed past 12 hours in response to intact dietary protein in LMB. The Walleye showed less noticeable differences between diets in the muscle FAA pool. One interesting observation was that at 12 h, the IDAA in the muscle was increasing in both groups, while the DAA was decreasing. Given the peak FAA times within the plasma in Walleye, and the peak appearance of FAA in muscle of cold-water species occurring at or after 12 h (Espe et al. 1993; Mente et al. 2003), it is possible that this increase in IDAA observed represents the initial appearance of dietary IDAA in the muscle. The muscle FAA pools of the Zebrafish were more responsive to the FAA50 diet than the CG diet. For both TFAA and IDAA the levels slightly peaked at 2 h, then decreased at 3 h, and then gradually increased over the 6 and 12 h time points. The FAA levels in the muscle of the CG fed Zebrafish did not show many changes over the postprandial sampling time. Similar to Walleye, given the 6 h peak in FAA levels in the

plasma in the ZF-CG group, it is feasible the FAA levels in the muscle peaked after the final 12 h sampling time.

Summary

The comparisons between the temporal dynamics of FAA absorption and distribution through the FAA pools within the body of LMB, Walleye, and Zebrafish highlight significant differences in the digestion and absorption of dietary protein as a result of differing physiological characteristics. Many of the results observed in this study support those presented in previous studies, with stronger grounds for comparison due to feeding with the same diets and assessing FAA dynamics at the same postprandial time points. In agreement with previous studies, the peaks of FAA within the body appeared earlier when stomach-possessing fish were fed with diets based on low molecular weight proteins, compared to diets based on intact protein (Yamada et al. 1981; Yamada et al. 1982; Ng et al. 1996; Cowey and Walton, 1988; Ambardekar et al. 2009). This rapid absorption of amino acids led to shorter, more drastic peaks, especially in the warm-water LMB. The slower digestion and release of amino acids for absorption led to more sustained peaks in FAA levels, and more correlations with the dietary IDAA profile in the plasma of the CG fed fish, compared to their FAA50 fed counterparts. Additionally, the slower metabolism of dietary protein associated with cool-water species led to more correlations with the dietary IDAA profile in the plasma of the Walleye, than the LMB or Zebrafish. The overall results observed in the plasma, liver, and muscle FAA levels suggests that neither diet was utilized efficiently in Zebrafish, with noticeably lower levels of dietary amino acids absorbed from the FAA50 diet. The differences in results between the stomach-possessing LMB and Walleye, and the stomachless Zebrafish highlight a reduced ability of stomachless fish to absorb and utilize dietary protein provided in FAA form. Future research should investigate more

postprandial sampling time points past the 12 h used in this study. This would allow for more observation of the duration of peaks and changes in FAA levels between the 12 h and starved (0 h) samples taken in this study, especially in cool-water species where it seems FAA peaks may begin or continue past 12 h after feeding. Additionally, although steps were taken to minimize the stress on fish in this study, a stress-induced metabolic response could not be completely ruled out as a factor in the immediate postprandial FAA dynamics of the Walleye. Further research associated with FAA dynamics should assess stress-related markers to identify any potential stress-induced effects on the postprandial FAA levels, especially in studies using force-feeding method. Overall, the results from this study provide for the first time a reference for temporal differences in FAA dynamics between species with different physiological characteristics when fed diets with intact protein or supplemented with crystalline amino acids.

CHAPTER 7

GENERAL CONCLUSIONS

Chapters 2, 3, and 4

Protein hydrolysates have been studied extensively as a means of supplying "pre-digested" protein to larval fish with underdeveloped digestive tracts. The results obtained from the dietary inclusion of hydrolysates have varied significantly based on the species tested and the different types of hydrolysates used. Given the species-specific amino acid requirements and digestive capacities of larval fish, an innovative approach was taken towards the production of protein hydrolysates that are tailored to the specific species they are intended for. Here we showed for the first time how species-specific hydrolysates can improve the utilization of dietary protein, and ultimately the growth and development of larval fish fed with formulated dry diets. Specifically, this research showed:

- The use of a same-species muscle hydrolysate was able to significantly improve the initial growth of larval Largemouth Bass (LMB), however, the growth was not improved over the duration of the larval stage. This was potentially due to an over-hydrolysis of the dietary hydrolysates, and a reduced utilization of low molecular weight protein as the LMB progressed through the larval stage. This study provides support for the initial inclusion of species-specific hydrolysates in diets for larval LMB, with a need to further identify the optimal molecular weight profile of dietary protein during the latter larval stages.
- The dietary inclusion of a muscle hydrolysate produced with species-specific muscle and endogenous enzymes significantly increased the growth of larval Walleye, compared to hydrolysates produced with inputs from Nile Tilapia. The species-specific hydrolysate

also supported postprandial absorption of the dietary amino acids. These results indicate that the use of both species-specific muscle and endogenous enzymes produces a protein hydrolysate that provides a more ideal dietary protein source as it pertains to amino acid absorption and growth in larval fish, than hydrolysates produced from other species.

- The whole-body Zebrafish hydrolysis method was able to produce a species-specific fishmeal hydrolysate and provides a simpler and more cost-effective means of producing hydrolysates for larval diets. The dietary inclusion of the hydrolyzed fishmeal did not result in significant differences in growth, but this was attributed to the potential adaptation of Zebrafish to dietary protein of different molecular forms. This study provides a practical method for producing dietary protein sources that are tailor-made for species-specific larvae and utilizes whole-body fish to reduce waste and potentially provide other species-specific nutrients in the body, in addition to the protein.

Chapter 5

Protein hydrolysates have been shown to act as a palatability enhancer, leading to increased intake of formulated diets, which is typically reduced in comparison to the intake of live feeds. Part of this enhanced palatability stems from high levels of FAA present in hydrolyzed protein. Protein hydrolysates have been found to increase intake even further when the FAA present leach out into the water, which triggers an olfactory response in fish and stimulates the detection and intake of feed. While the effect of hydrolysates on feed intake has been studied extensively, the effect of the dietary amino acid profile on feed intake has remained unclear. Given that one of the major focuses for improving the use of dietary protein hydrolysates is to ensure that it fulfills the specific amino acid requirements of each species, it was important to understand how fish respond to diets that meet these requirements vs diets that

are deficient in some amino acids, especially IDAA. We provided for the first-time evidence for physiological and behavioral responses to dietary IDAA deficiencies in stomachless fish, and the impact of those deficiencies on the acceptance and utilization of formulated dry diets. Specifically, the research showed:

- The results on Zebrafish showed that although the singular deficiency of the three main limiting amino acids did not induce significant changes in feed intake, the combined deficiency of the three IDAA (lysine, methionine, and threonine) significantly increased the feed intake of juvenile Zebrafish. This increased feed intake prevented the IDAA deficiencies from significantly reducing growth, however as a result, the feeding efficiency was also reduced. The reduced feeding efficiency could be due to a decrease in protein synthesis as a result of an imbalanced FAA pool, signified by a significantly lower postprandial level of IDAA in the muscle FAA pool, but a significantly higher level of DAA. The analysis of appetite-regulating hormones suggests that an upregulation of orexigenic hormone *NPY* might play a role behind the increased feed intake in response to this deficiency. The outcomes of this study provide insight into the behavioral and physiological response to dietary amino acid imbalances of stomachless fish.

Chapter 6

The analysis of postprandial free amino acid (FAA) pools has been extensively studied as a tool for assessing the absorption and utilization of dietary amino acids in fish. The FAA dynamics provide insight into the quality and digestibility of the dietary protein and have been shown to vary based on the molecular form of dietary protein and the physiological characteristics of the fish. Here we established for the first time, a reference for the FAA

dynamics of three species with variable physiological characteristics, when fed with the same intact or FAA-based diets. Specifically, the research showed:

- The postprandial FAA dynamics in the plasma, liver, and muscle were significantly different between the LMB, Walleye, and Zebrafish. Within the stomach-possessing species, the dietary amino acids from the FAA-based diets were absorbed more rapidly than those from the intact-based diet. This resulted in fewer correlations with the dietary IDAA profiles, indicating the rapid influx of dietary FAA was less balanced than that of intact protein. The absorption of FAA in the cool-water Walleye was more gradual and prolonged than the warm-water LMB, leading to more significant correlations with the dietary IDAA and more sustained peaks. These trends were not seen in stomachless species, as peaks of FAA in Zebrafish typically occurred at the same time between diets but were lower after feeding with dietary FAA. The postprandial FAA patterns observed in this study provide a reference for variations in FAA dynamics between species with different physiological characteristics when fed diets based on FAA or intact protein.

Final remarks

The production of a dry diet suitable to sustain high levels of growth and survival in larval fish would ease the reliance on live feeds and increase the potential for the sustainable intensive rearing of larvae of more species. The results of the studies conducted provide significant support for the use of species-specific protein hydrolysates as an ideal protein source to produce formulated diets that are able to replace live feed in first-feeding larvae. The novel approaches presented provide cost-effective methods for the production of these species-specific hydrolysates and can be tailored to a wide range of aquaculture species. The use of these

hydrolysates can be advanced with further research into the optimal hydrolysis level of the dietary protein, and into means of optimizing the physical properties of these diets to reduce excess leaching and improve the delivery of dietary protein. With these advancements, the innovative production of species-specific hydrolysates can increase the consistency of juvenile production and economic sustainability of intensive larval rearing.

Further insight into the physiological and behavioral effects of the amino acid profile and molecular weight profile of the dietary protein was also provided in this dissertation. A response in feed intake to IDAA deficiencies was observed, and evidence of the nutrient-sensing pathway behind this response was found in the brain of the fish. Additionally, the research conducted provides a reference for the absorption dynamics of dietary amino acids from varying molecular weight profiles in species with different physiological characteristics.

Ultimately, the findings of this research contribute to the development of larval diets that can release the limitations of growth placed on the aquaculture industry particularly within the hatchery sector. Additionally, this research provides further understanding of dietary protein utilization and delivers new fish nutrition knowledge that will undoubtedly contribute to the aquaculture industry as a whole.

EXHIBITS

TABLES

Table 2.1. Diet formulations for feeding trial.

Ingredients (%)	**Intact**	**A**	**B**	**C**	**D**
Fish meal (Intact)	74.00	37.00	37.00	37.00	37.00
Hydrolysate 1.5h	-	12.30	3.70	7.40	22.20
Hydrolysate 3h	-	12.30	11.10	22.20	11.10
Hydrolysate 6h	-	12.30	22.20	7.40	3.70
CPSP 90[1]	5.00	5.00	5.00	5.00	5.00
Krill Meal[2]	5.00	5.00	5.00	5.00	5.00
Fish oil[3]	4.00	4.00	4.00	4.00	4.00
Lecithin[4]	4.00	4.00	4.00	4.00	4.00
Mineral mix[5]	3.00	3.00	3.00	3.00	3.00
Vitamin mix[6]	3.00	3.00	3.00	3.00	3.00
$CaHPO_4$	1.00	1.00	1.00	1.00	1.00
Taurine	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.10	0.10	0.10	0.10	0.10
Vitamin C[7]	0.05	0.05	0.05	0.05	0.05
Proximate Comp. (%)					
Crude Protein (*N* x 6.25)	60.97 (± 0.08)	60.99 (± 0.17)	60.96 (± 0.20)	61.02 (± 0.03)	60.96 (± 0.19)
Lipids	18.05 (± 0.18)	18.06 (± 0.08)	18.00 (± 0.10)	17.98 (± 0.16)	17.97 (± 0.15)
Ash	12.01 (± 0.07)	11.99 (± 0.17)	12.02 (± 0.15)	12.07 (± 0.13)	12.06 (± 0.11)

[1] Soluble fish protein hydrolysate (Sopropeche S.A., Boulogne Sur Mer, France).

[2] Proccesed Euphausia superba (Florida Aqua Farms, Dade City, FL, USA).

[3] Cod liver oil (MP Biomedicals, Solon, OH, USA).

[4] Yelkin TS lecithin (Ingredi Co., Baltimore, MD, USA).

[5] Bernhart-Tomarelli mineral mix with 5ppm selenium in a form of sodium selenite (Dyets, Bethlehem, PA, USA).

[6] Custom Vitamin Mixture (mg/kg diet) Thiamin HCl, 4.56; Riboflavin, 4.80; Pyridoxine HCl, 6.86; Niacin, 10.90; D-Calcium Pantothenate, 50.56; Folic Acid, 1.26; D-Biotin, 0.16; Vitamin B12 (0.1%), 20.00; Vitamin A Palmitate (500,000 IU/g), 9.66; Vitamin D3 (400,000 IU/g), 8.26; Vitamin E Acetate (500 IU/g), 132.00; Menadione Sodium Bisulfite, 2.36; Inositol, 500 (Dyets, Bethlehem, PA, USA).

[7] L-Ascorbyl-2-Polyphosphate (Argent Aquaculture, Redmond, WA, USA).

Table 2.2 Dietary amino acid composition.

Amino acid composition (%)	Intact	A	B	C	D
Alanine	3.90 (± 0.10)	3.73 (± 0.01)	3.72 (± 0.10)	3.73 (± 0.09)	3.89 (± 0.10)
Arginine	3.54 (± 0.08)	3.38 (± 0.02)	3.41 (± 0.08)	3.38 (± 0.08)	3.49 (± 0.06)
Aspartic Acid	5.69 (± 0.11)	5.49 (± 0.02)	5.49 (± 0.10)	5.53 (± 0.17)	5.35 (± 0.09)
Cysteine	0.57 (± 0.02)	0.54 (± 0.02)	0.57 (± 0.01)	0.55 (± 0.04)	0.50 (± 0.04)
Glutamic Acid	8.02 (± 0.19)	7.76 (± 0.06)	7.64 (± 0.20)	7.70 (± 0.16)	7.84 (± 0.10)
Glycine	4.73 (± 0.20)	4.33 (± 0.08)	4.48 (± 0.15)	4.45 (± 0.15)	4.70 (± 0.18)
Histidine	1.35 (± 0.02)	1.35 (± 0.02)	1.32 (± 0.02)	1.32 (± 0.05)	1.26 (0.05)
Hydroxyproline	0.87 (± 0.07)	0.74 (± 0.05)	0.83 (± 0.04)	0.79 (± 0.18)	1.13 (± 0.20)
Isoleucine	2.65 (± 0.05)	2.62 (± 0.03)	2.56 (± 0.05)	2.55 (± 0.11)	2.43 (± 0.10)
Leucine	4.29 (± 0.09)	4.32 (± 0.09)	4.15 (± 0.08)	4.18 (± 0.15)	3.99 (± 0.17)
Lysine	4.81 (± 0.10)	4.85 (± 0.11)	4.65 (± 0.09)	4.65 (± 0.18)	4.45 (± 0.20)
Methionine	1.57 (± 0.04)	1.51 (± 0.01)	1.49 (± 0.04)	1.52 (± 0.05)	1.47 (± 0.02)
Phenylalanine	2.27 (± 0.04)	2.26 (± 0.04)	2.19 (± 0.04)	2.21 (± 0.05)	2.13 (± 0.02)
Proline	2.71 (± 0.14)	2.42 (± 0.07)	2.55 (± 0.09)	2.54 (±0.17)	2.89 (± 0.24)
Serine	2.12 (± 0.04)	2.04 (± 0.03)	2.07 (± 0.03)	2.10 (± 0.04)	2.05 (± 0.01)
Taurine	1.95 (± 0.27)	2.50 (± 0.17)	2.19 (± 0.15)	2.23 (± 0.14)	2.06 (± 0.23)
Threonine	2.49 (± 0.05)	2.40 (± 0.03)	2.43 (± 0.03)	2.46 (± 0.07)	2.36 (± 0.03)
Tryptophan	0.62 (± 0.08)	0.54 (± 0.05)	0.45 (± 0.08)	0.52 (± 0.08)	0.46 (± 0.05)
Tyrosine	1.79 (± 0.06)	1.86 (± 0.06)	1.73 (± 0.03)	1.77 (± 0.06)	1.68 (± 0.09)
Valine	2.98 (± 0.05)	2.94 (± 0.03)	2.88 (± 0.05)	2.90 (± 0.09)	2.81 (± 0.07)

Diets were analyzed in triplicates. The values presented are the mean (± S.D.).

Table 2.3. Primers used to amplify target genes

	Primer	Nucleotide sequence (5'- 3')
PepT1	Forward	AAGCTGGGACCGAGAAGATT
	Reverse	CGGCCAATAAAGTGGTTTCA
eEF1a1	Forward	GTTGCTGCTGGTGTTGGTGAG
	Reverse	GAAACGCTTCTGGCTGTAAGG
α-tubulin	Forward	GACTCGACCACAAGTTTGACC
	Reverse	GTTCCCACCTCTTCGTAATCC
β-actin	Forward	GTATTGTCATGGACTCTGGTG
	Reverse	ACGTACGATTTCACGCTCAGC

Table 2.4**.** Diet effect on growth and survival. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-Way ANOVA and a Tukey test with a p value < 0.05.

Group	Avg. Weight (mg)	Avg. Total Length (mm)	Survival (%)
LF-Intact	79.50^{c} (± 30.00)	19.93^{d} (± 2.49)	46.29^{c} (± 9.20)
LF-Hydro	70.67^{bc} (± 23.71)	18.60^{c} (± 1.95)	33.78^{b} (± 6.72)
Intact	51.67^{ac} (± 14.74)	16.55^{b} (± 2.25)	23.94^{b} (± 8.69)
Hydro-A	44.33^{ab} (± 4.04)	16.35^{ab} (± 2.38)	11.69^{a} (± 2.11)
Hydro-BCD	35.83^{a} (± 6.37)	15.78^{ab} (± 2.05)	11.33^{a} (± 4.89)
Hydro-DCB	41.00^{a} (± 7.81)	15.31^{a} (± 2.88)	9.96^{a} (± 2.85)
Artemia	72.32^{bc} (± 10.98)	17.74^{c} (± 1.55)	86.89^{d} (± 1.81)

Table 2.5. Postprandial FAA composition in muscle. The values presented are postprandial levels, 2 h after feeding. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-way ANOVA and an LSD test with a p value < 0.05. Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids.

Amino Acid (μM/g)	LF-Intact	LF-Hydro	Intact	Hydro-A	Hydro-BCD	Hydro-DCB	Artemia
Aspartic Acid	0.64^{ab} (± 0.02)	0.65^{ab} (± 0.01)	0.61^{ab} (± 0.06)	0.61^{ab} (± 0.05)	0.63^{ab} (± 0.01)	0.70^{b} (± 0.05)	0.53^{a} (± 0.08)
Glutamic Acid	1.56^{bc} (± 0.08)	1.63^{c} (± 0.05)	1.41^{ac} (± 0.05)	1.14^{a} (± 0.11)	1.40^{ac} (± 0.07)	1.35^{ac} (± 0.03)	1.25^{ab} (± 0.29)
Asparagine	0.38^{a} (± 0.04)	0.50^{ab} (± 0.02)	0.54^{ab} (± 0.08)	0.46^{ab} (± 0.09)	0.53^{ab} (± 0.04)	0.57^{b} (± 0.08)	0.45^{ab} (± 0.05)
Serine	0.55^{a} (± 0.11)	0.74^{ab} (± 0.10)	0.58^{a} (± 0.13)	0.60^{a} (± 0.12)	0.88^{b} (± 0.07)	0.93^{b} (± 0.04)	0.55^{a} (± 0.07)
Glutamine	0.63^{ab} (± 0.04)	0.72^{ab} (± 0.03)	0.79^{b} (± 0.05)	0.53^{a} (± 0.09)	0.71^{ab} (± 0.06)	0.76^{ab} (± 0.17)	0.64^{ab} (± 0.05)
Histidine	0.73^{b} (± 0.12)	0.69^{b} (± 0.05)	0.61^{b} (± 0.14)	0.61^{b} (± 0.01)	0.56^{b} (± 0.04)	0.60^{b} (± 0.01)	0.28^{a} (± 0.11)
Glycine	1.91^{bc} (± 0.36)	2.55^{c} (± 0.15)	1.35^{b} (± 0.44)	1.31^{ab} (± 0.22)	1.69^{b} (± 0.30)	1.75^{b} (± 0.03)	0.61^{a} (± 0.04)
Threonine	0.70^{b} (± 0.11)	0.94^{c} (± 0.03)	0.61^{b} (± 0.09)	0.58^{ab} (± 0.09)	0.73^{b} (± 0.06)	0.68^{b} (± 0.02)	0.41^{a} (± 0.08)
Arginine	0.09^{b} (± 0.01)	0.12^{c} (± 0.01)	0.10^{b} (± 0.00)	0.09^{b} (± 0.01)	0.11^{bc} (± 0.01)	0.10^{b} (± 0.01)	0.04^{a} (± 0.01)
Alanine	0.83^{abc} (± 0.19)	1.06^{bd} (± 0.10)	0.82^{ab} (± 0.23)	1.00^{bc} (± 0.13)	1.22^{cd} (± 0.03)	1.41^{d} (± 0.02)	0.54^{a} (± 0.13)
Tyrosine	0.20^{bc} (± 0.01)	0.25^{d} (± 0.01)	0.19^{bc} (± 0.02)	0.17^{b} (± 0.03)	0.22^{cd} (± 0.01)	0.19^{bc} (± 0.01)	0.09^{a} (± 0.02)
Lysine	1.11^{b} (± 0.09)	1.24^{b} (± 0.03)	1.21^{b} (± 0.07)	0.98^{ab} (± 0.06)	1.16^{b} (± 0.04)	1.12^{b} (± 0.07)	0.73^{a} (± 0.19)
Methionine	1.57^{a} (± 0.06)	1.87^{ab} (± 0.18)	1.74^{ab} (± 0.18)	2.21^{b} (± 0.20)	1.87^{ab} (± 0.08)	1.95^{ab} (± 0.11)	2.06^{ab} (± 0.43)
Valine	1.95 (± 0.07)	2.32 (± 0.15)	2.01 (± 0.05)	2.34 (± 0.52)	1.99 (± 0.09)	2.29 (± 0.10)	2.41 (± 0.13)

Cysteine *not detected	-	-	-	-	-	-	-
Tryptophan	0.12^{ab} (± 0.03)	0.11^{a} (± 0.01)	0.12^{ab} (± 0.00)	0.08^{a} (± 0.01)	0.16^{b} (± 0.03)	0.12^{ab} (± 0.00)	0.10^{a} (± 0.01)
Phenylalanine	0.28^{b} (± 0.02)	0.30^{b} (± 0.02)	0.33^{bc} (± 0.02)	0.28^{b} (± 0.01)	0.39^{c} (± 0.04)	0.33^{b} (± 0.01)	0.19^{a} (± 0.02)
Isoleucine	0.24 (± 0.17)	0.15 (± 0.03)	0.34 (± 0.22)	0.11 (± 0.03)	0.19 (± 0.04)	0.14 (± 0.03)	0.16 (± 0.02)
Leucine	0.24 (± 0.14)	0.38 (± 0.05)	0.33 (± 0.21)	0.35 (± 0.05)	0.44 (± 0.05)	0.40 (± 0.04)	0.25 (± 0.04)
Proline	0.35^{a} (± 0.02)	0.47^{ab} (± 0.07)	0.49^{ab} (± 0.17)	0.87^{cd} (± 0.03)	0.71^{bc} (± 0.08)	1.07^{d} (± 0.04)	0.58^{ab} (± 0.12)
IDAA	7.03^{ab} (± 0.28)	8.11^{c} (± 0.26)	7.40^{ac} (± 0.36)	7.65^{ac} (± 0.63)	7.60^{ac} (± 0.29)	7.74^{bc} (± 0.13)	6.62^{a} (± 0.45)
DAA	7.06^{ad} (± 0.67)	8.57^{cd} (± 0.16)	6.78^{ac} (± 1.10)	6.70^{ab} (± 0.84)	7.99^{bcd} (± 0.34)	8.73^{d} (± 0.31)	5.24^{a} (± 0.71)
TFAA	14.10^{ab} (± 0.95)	16.68^{c} (± 0.41)	14.18^{ac} (± 1.33)	14.35^{ac} (± 1.10)	15.59^{bc} (± 0.60)	16.46^{bc} (± 0.37)	11.87^{a} (± 1.11)

Table 2.6. Basal FAA composition in muscle. The values presented are postprandial levels, 24 h after feeding. Values are presented as means (± S.D.). Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids.

Amino Acid (μM/g)	LF-Intact	LF-Hydro	Intact	Hydro-A	Hydro-BCD	Hydro-DCB	Artemia
Aspartic Acid	0.62 (± 0.02)	0.70 (± 0.06)	0.75 (± 0.03)	0.73 (± 0.02)	0.72 (± 0.06)	0.66 (± 0.01)	0.46 (± 0.01)
Glutamic Acid	1.34 (± 0.05)	1.29 (± 0.09)	1.45 (± 0.09)	1.25 (± 0.03)	1.45 (± 0.08)	1.19 (± 0.06)	0.93 (± 0.05)
Asparagine	0.45 (± 0.03)	0.42 (± 0.04)	0.57 (± 0.04)	0.50 (± 0.01)	0.51 (± 0.06)	0.43 (± 0.01)	0.59 (± 0.09)
Serine	0.30 (± 0.04)	0.29 (± 0.01)	0.51 (± 0.06)	0.52 (± 0.05)	0.66 (± 0.09)	0.42 (± 0.02)	0.72 (± 0.13)
Glutamine	0.55 (± 0.03)	0.52 (± 0.04)	0.83 (± 0.04)	0.68 (± 0.02)	0.86 (± 0.09)	0.52 (± 0.02)	0.70 (± 0.07)
Histidine	0.73 (± 0.07)	0.54 (± 0.09)	0.56 (± 0.07)	0.42 (± 0.07)	0.41 (± 0.03)	0.34 (± 0.02)	0.25 (± 0.08)
Glycine	1.89 (± 0.45)	2.64 (± 0.49)	1.60 (± 0.26)	1.43 (± 0.25)	1.53 (± 0.23)	1.47 (± 0.14)	0.70 (± 0.13)
Threonine	0.64 (± 0.06)	0.88 (± 0.13)	0.62 (± 0.04)	0.54 (± 0.06)	0.59 (± 0.04)	0.51 (± 0.04)	0.31 (± 0.04)
Arginine	0.07 (± 0.01)	0.05 (± 0.00)	0.07 (± 0.01)	0.06 (± 0.01)	0.05 (± 0.00)	0.04 (± 0.00)	0.03 (± 0.00)
Alanine	0.50 (± 0.06)	0.36 (± 0.04)	0.50 (± 0.04)	0.57 (± 0.07)	0.51 (± 0.11)	0.40 (± 0.01)	0.84 (± 0.17)
Tyrosine	0.13 (± 0.02)	0.19 (± 0.02)	0.16 (± 0.01)	0.14 (± 0.01)	0.18 (± 0.00)	0.15 (± 0.01)	0.04 (± 0.00)
Lysine	0.71 (± 0.06)	0.79 (± 0.05)	0.70 (± 0.04)	0.60 (± 0.05)	0.53 (± 0.02)	0.45 (± 0.05)	0.24 (± 0.02)
Methionine	0.34 (± 0.04)	0.34 (± 0.04)	0.39 (± 0.03)	0.34 (± 0.05)	0.33 (± 0.07)	0.55 (± 0.04)	0.40 (± 0.01)
Valine	1.91 (± 0.09)	1.85 (± 0.06)	1.83 (± 0.08)	1.95 (± 0.08)	1.63 (± 0.04)	2.17 (± 0.12)	1.80 (± 0.03)
Cysteine *not detected	-	-	-	-	-	-	

Tryptophan	0.05 (± 0.00)	0.06 (± 0.00)	0.08 (± 0.00)	0.08 (± 0.00)	0.10 (± 0.01)	0.08 (± 0.00)	0.06 (± 0.01)
Phenylalanine	0.18 (± 0.01)	0.21 (± 0.02)	0.23 (± 0.03)	0.22 (± 0.00)	0.23 (± 0.01)	0.22 (± 0.01)	0.12 (± 0.00)
Isoleucine	0.10 (± 0.00)	0.06 (± 0.01)	0.10 (± 0.01)	0.10 (± 0.01)	0.05 (± 0.02)	0.03 (± 0.00)	0.04 (± 0.00)
Leucine	0.21 (± 0.01)	0.17 (± 0.02)	0.24 (± 0.01)	0.20 (± 0.01)	0.14 (± 0.01)	0.12 (± 0.01)	0.09 (± 0.00)
Proline	0.54 (± 0.06)	0.74 (± 0.13)	0.66 (± 0.03)	1.05 (± 0.25)	0.98 (± 0.16)	1.08 (± 0.10)	1.33 (± 0.07)
IDAA	4.94 (± 0.26)	4.96 (± 0.24)	4.82 (± 0.26)	4.51 (± 0.10)	4.05 (± 0.13)	4.51 (± 0.02)	3.35 (± 0.11)
DAA	6.30 (± 0.54)	7.16 (± 0.68)	7.04 (± 0.28)	6.86 (± 0.63)	7.39 (± 0.31)	6.36 (± 0.27)	6.32 (± 0.66)
TFAA	11.24 (± 0.80)	12.12 (± 0.92)	11.86 (± 0.53)	11.37 (± 0.63)	11.44 (± 0.41)	10.85 (± 0.25)	9.66 (± 0.77)

Table 3.1. Diet formulations for Walleye feeding trial.

Ingredients (%)	**Diet**
Intact[a]	37.00
Hydrolysate[b]	37.00
Krill Meal[c]	5.00
Fish oil[d]	9.00
Lecithin[e]	4.00
Mineral mix[f]	3.00
Vitamin mix[g]	3.00
$CaHPO_4$	1.00
Taurine	1.00
Choline chloride	0.10
Vitamin C[h]	0.05
Sum	100

[a] Intact muscle protein that did not undergo incubated hydrolysis.
[b] Hydrolyzed muscle protein produced with *in vitro* hydrolysis method.
[c] Processed *Euphausia superba* (Florida Aqua Farms, Dade City, FL, USA).
[d] Cod liver oil (MP Biomedicals, Solon, OH, USA).
[e] Refined soy lecithin (MP Biomedicals, Solon, OH, USA).
[f] Bernhart-Tomarelli mineral mix with 5ppm selenium in a form of sodium selenite (Dyets, Bethlehem, PA, USA).
[g] Custom Vitamin Mixture (mg/kg diet) Thiamin HCl, 6.84; Riboflavin, 7.2; Pyridoxine HCl, 10.29; Niacin, 16.35; D-Calcium Pantothenate, 75.84; Folic Acid, 1.89; D-Biotin, 0.24; Vitamin B12 (0.1%), 30; Vitamin A Palmitate (500,000 IU/g), 14.49; Vitamin D3 (400,000 IU/g), 12.39; Vitamin E Acetate (500 IU/g), 198; Menadione Sodium Bisulfite, 3.54; Inositol, 750 (Dyets, Bethlehem, PA, USA).
[h] L-Ascorbyl-2-Polyphosphate (Argent Aquaculture, Redmond, WA, USA).

Table 3.2. Analyzed composition of formulated diets. Diets were analyzed in triplicates and the values are presented as mean (± S. D.)

Analyzed Composition (g/100g) Dry Matter	**W-W**	**W-T**	**T-W**	**T-T**
Crude Protein (N x 6.25)	60.23 (± 0.09)	60.20 (± 0.15)	59.92 (± 0.25)	59.93 (± 0.05)
Crude Lipids	15.30 (± 0.05)	15.47 (± 0.02)	15.66 (± 0.02)	15.08 (± 0.08)
Ash	13.48 (± 0.21)	13.45 (± 0.09)	13.82 (± 0.09)	13.74 (± 0.45)
Alanine	3.70 (± 0.02)	3.86 (± 0.13)	3.73 (± 0.01)	3.77 (± 0.09)
Arginine	3.40 (± 0.03)	3.08 (± 0.12)	3.57 (± 0.01)	3.17 (± 0.09)
Aspartic Acid	5.58 (± 0.02)	5.63 (± 0.04)	5.30 (± 0.07)	5.45 (± 0.09)
Cysteine	0.59 (± 0.02)	0.63 (± 0.00)	0.53 (± 0.01)	0.58 (± 0.02)
Glutamic Acid	7.97 (± 0.24)	7.81 (± 0.10)	7.71 (± 0.03)	7.97 (± 0.09)
Glycine	3.86 (± 0.04)	4.20 (± 0.28)	4.51 (± 0.00)	4.35 (± 0.32)
Histidine	1.49 (± 0.02)	1.43 (± 0.03)	1.27 (± 0.01)	1.36 (± 0.03)
Hydroxyproline	0.60 (± 0.02)	0.65 (± 0.01)	1.13 (± 0.11)	0.94 (± 0.14)
Isoleucine	2.65 (± 0.03)	2.66 (± 0.04)	2.49 (± 0.06)	2.59 (± 0.08)
Leucine	4.31 (± 0.03)	4.31 (± 0.05)	4.07 (± 0.02)	4.23 (± 0.09)
Lysine	5.04 (± 0.03)	4.61 (± 0.07)	4.52 (± 0.01)	4.28 (± 0.08)
Methionine	1.69 (± 0.01)	1.70 (± 0.00)	1.45 (± 0.03)	1.51 (± 0.02)
Phenylalanine	2.32 (± 0.02)	2.34 (± 0.05)	2.17 (± 0.03)	2.28 (± 0.04)
Proline	2.34 (± 0.03)	2.50 (± 0.08)	2.70 (± 0.04)	2.63 (± 0.14)
Serine	2.26 (± 0.11)	2.24 (± 0.05)	2.02 (± 0.06)	2.00 (± 0.02)
Taurine	1.53 (± 0.01)	1.54 (± 0.03)	1.87 (± 0.03)	1.86 (± 0.13)
Threonine	2.51 (± 0.00)	2.56 (± 0.01)	2.41 (± 0.03)	2.47 (± 0.03)
Tryptophan	0.61 (± 0.03)	0.64 (± 0.03)	0.54 (± 0.01)	0.61 (± 0.03)
Tyrosine	1.79 (± 0.01)	1.80 (± 0.05)	1.62 (± 0.11)	1.73 (± 0.04)
Valine	2.83 (± 0.04)	2.91 (± 0.02)	2.73 (± 0.04)	2.84 (± 0.05)
Sum	57.06 (± 0.24)	57.11 (± 0.20)	56.33 (± 0.27)	56.63 (± 0.41)

W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes.

Table 3.3. Primers used to amplify target genes.

Gene	Primer	Nucleotide sequence (5'- 3')
PepT1	Forward	CACACCCAGCAGAAGTGCTACT
	Reverse	ACAATCAGAGCTACCACCATGAGA
	Probe	FAM-ACTGGCCTTTGGTGTCCCCGC-NFQ
eEF1a1	Forward	GGAAATCCGTCGTGGATATGTG
	Reverse	TGACCTGGGCGTTGAAGTTG
	Probe	FAM-CTGGCGACAGCAAGAACGACCCACC-NFQ
α-tubulin	Forward	ACCAACCTCAACAGGCTAATTGG
	Reverse	GAGGGCACCATCGAAACGA
	Probe	FAM-CAGATTGTGTCCTCCATCACTGCCTCCC-NFQ
β-actin	Forward	CCCTCTTCCAGCCTTCCTT
	Reverse	GTAGGTGGTCTCGTGGATTCC
	Probe	FAM-CCTCGGTATGGAGTCCTG-NFQ

Table 3.4. Diet effect on growth and survival. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-Way ANOVA and a Tukey test at $p < 0.05$.

Group	Avg. Weight (mg)	Survival (%)
Artemia	61.01^{a} (± 12.47)	53.63^{b} (± 11.43)
Commercial	57.35^{a} (± 6.18)	39.41^{b} (± 1.39)
W-W	89.84^{b} (± 6.09)	16.87^{a} (± 5.71)
W-T	62.83^{a} (± 4.03)	13.95^{a} (± 2.41)
T-W	61.22^{a} (± 9.61)	20.98^{a} (± 3.16)
T-T	54.70^{a} (± 8.62)	21.69^{a} (± 4.80)

W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes.

Table 3.5. Results of Two-way ANOVA. In the presence of no significant (NS) interaction effect, the p-values for each main effect are presented. Effects were determined to be significant at $p < 0.05$ and are indicated with an asterisk.

Parameter	Group	Mean (± S.D.)	Factor	p-value
Avg. Weight (mg)	W-W	89.84 (± 6.09)	Muscle Source	-
	W-T	62.83 (± 4.03)	Enzyme Source	-
	T-W	61.22 (± 9.61)	Interaction*	0.0437
	T-T	54.70 (± 8.62)		
Survival (%)	W-W	16.87 (± 5.71)	Muscle Source*	0.0344
	W-T	13.95 (± 2.41)	Enzyme Source	0.6553
	T-W	20.98 (± 3.16)	Interaction	NS
	T-T	21.69 (± 4.80)		
Total Occurrence of Skeletal Deformities (%)	W-W	4.00 (± 1.00)	Muscle Source	0.2126
	W-T	5.67 (± 1.53)	Enzyme Source	0.3406
	T-W	6.00 (± 1.73)	Interaction	NS
	T-T	6.33 (± 2.52)		
Postprandial (2 h) PepT1 Expression (Fold Change)	W-W	2.05 (± 0.61)	Muscle Source	0.3442
	W-T	2.03 (± 0.69)	Enzyme Source	0.4652
	T-W	2.29 (± 0.58)	Interaction	NS
	T-T	4.03 (± 2.02)		
Postprandial (2 h) Total FAA Level in Muscle (μM/g)	W-W	25.69 (± 2.00)	Muscle Source*	0.0013
	W-T	20.17 (± 0.84)	Enzyme Source*	0.0012
	T-W	20.22 (± 1.49)	Interaction	NS
	T-T	16.30 (± 2.43)		
Postprandial (2 h) Total IDAA Level in Muscle (μM/g)	W-W	14.87 (± 1.23)	Muscle Source*	0.0008
	W-T	10.61 (± 0.41)	Enzyme Source*	0.0003
	T-W	11.02 (± 0.72)	Interaction	NS
	T-T	8.78 (± 0.90)		
Postprandial (2 h) Total DAA Level in Muscle (μM/g)	W-W	10.82 (± 0.77)	Muscle Source*	0.0085
	W-T	9.56 (± 0.43)	Enzyme Source*	0.0243
	T-W	9.20 (± 0.87)	Interaction	NS
	T-T	7.52 (± 1.56)		

W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes.

Table 3.6. Postprandial FAA composition in muscle. The values presented are postprandial levels, 2 h after feeding. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-way ANOVA and an LSD test with a p value < 0.05. Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids

Amino Acid (μM/g)	Artemia	Commercial	W-W	W-T	T-W	T-T
Aspartic Acid	1.00 (± 0.16)	1.41 (± 0.25)	1.03 (± 0.10)	1.16 (± 0.04)	1.09 (± 0.14)	1.00 (± 0.19)
Glutamic Acid	1.78^{ab} (± 0.18)	2.14^{b} (± 0.28)	1.84^{ab} (± 0.15)	1.86^{ab} (± 0.03)	1.80^{ab} (± 0.19)	1.45^{a} (± 0.21)
Asparagine	0.44^{c} (± 0.12)	0.36^{bc} (± 0.09)	0.20^{ab} (± 0.01)	0.17^{a} (± 0.02)	0.19^{ab} (± 0.03)	0.15^{a} (± 0.06)
Serine	1.55^{b} (± 0.50)	1.20^{ab} (± 0.33)	0.68^{a} (± 0.04)	0.84^{ab} (± 0.06)	0.70^{a} (± 0.11)	0.53^{a} (± 0.18)
Glutamine	0.50^{ab} (± 0.02)	0.58^{b} (± 0.04)	0.63^{b} (± 0.03)	0.52^{ab} (± 0.03)	0.59^{b} (± 0.08)	0.38^{a} (± 0.08)
Histidine	1.77^{a} (± 0.48)	1.73^{a} (± 0.28)	2.66^{b} (± 0.04)	1.53^{a} (± 0.11)	1.63^{a} (± 0.28)	1.07^{a} (± 0.31)
Glycine	1.96^{a} (± 0.67)	1.75^{a} (± 0.44)	3.36^{b} (± 0.05)	1.95^{a} (± 0.12)	1.91^{a} (± 0.24)	1.86^{a} (± 0.41)
Threonine	0.90^{a} (± 0.03)	1.53^{b} (± 0.30)	1.21^{ab} (± 0.07)	1.26^{ab} (± 0.06)	1.30^{ab} (± 0.11)	0.89^{a} (± 0.22)
Arginine	0.29^{a} (± 0.03)	0.45^{ab} (± 0.08)	1.04^{c} (± 0.17)	0.53^{b} (± 0.03)	0.63^{b} (± 0.02)	0.44^{ab} (± 0.08)
Alanine	1.80^{bc} (± 0.51)	2.43^{c} (± 0.27)	1.49^{ab} (± 0.16)	1.64^{ab} (± 0.17)	1.37^{ab} (± 0.12)	0.89^{a} (± 0.25)
Tyrosine	0.45^{a} (± 0.02)	0.46^{a} (± 0.08)	0.79^{b} (± 0.15)	0.62^{ab} (± 0.06)	0.55^{ab} (± 0.03)	0.54^{ab} (± 0.14)
Lysine	0.55^{a} (± 0.14)	0.93^{ab} (± 0.22)	3.57^{d} (± 0.53)	1.88^{c} (± 0.30)	1.77^{c} (± 0.12)	1.59^{bc} (± 0.09)
Methionine	1.61^{a} (± 0.16)	1.68^{a} (± 0.15)	2.83^{c} (± 0.26)	2.10^{ab} (± 0.12)	2.40^{bc} (± 0.30)	2.06^{ab} (± 0.13)
Valine	1.44^{a} (± 0.07)	1.49^{a} (± 0.09)	2.40^{c} (± 0.07)	2.03^{b} (± 0.10)	2.39^{c} (± 0.12)	2.04^{b} (± 0.11)

Cysteine *Not detected	-	-	-	-	-	-
Tryptophan	0.03^{ab} (± 0.01)	0.02^{ab} (± 0.01)	0.04^{b} (± 0.00)	0.04^{b} (± 0.00)	0.03^{ab} (± 0.00)	0.02^{a} (± 0.01)
Phenylalanine	0.19^{a} (± 0.03)	0.17^{a} (± 0.02)	0.25^{ab} (± 0.03)	0.27^{b} (± 0.02)	0.19^{ab} (± 0.03)	0.20^{ab} (± 0.04)
Isoleucine	0.18^{ab} (± 0.02)	0.23^{bc} (± 0.04)	0.28^{cd} (± 0.03)	0.32^{d} (± 0.00)	0.21^{ac} (± 0.02)	0.14^{a} (± 0.03)
Leucine	0.32^{a} (± 0.07)	0.47^{ab} (± 0.10)	0.59^{bc} (± 0.05)	0.66^{c} (± 0.00)	0.47^{ab} (± 0.05)	0.34^{a} (± 0.07)
Proline	0.46^{a} (± 0.06)	0.74^{ac} (± 0.11)	0.80^{bc} (± 0.10)	0.80^{bc} (± 0.02)	1.02^{c} (± 0.19)	0.71^{ab} (± 0.10)
IDAA	7.27^{a} (± 0.47)	8.71^{ab} (± 0.63)	14.87^{d} (± 1.23)	10.61^{bc} (± 0.41)	11.02^{c} (± 0.72)	8.78^{ab} (± 0.90)
DAA	9.94^{ab} (± 1.36)	11.09^{b} (± 0.60)	10.82^{b} (± 0.77)	9.56^{ab} (± 0.43)	9.20^{ab} (± 0.87)	7.52^{a} (± 1.56)
TFAA	17.21^{a} (± 1.79)	19.80^{a} (± 0.03)	25.69^{b} (± 2.00)	20.17^{a} (± 0.84)	20.22^{a} (± 1.49)	16.30^{a} (± 2.43)

W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes.

Table 3.7. Basal FAA composition in muscle. The values presented are basal levels, 24 h after feeding. Values are presented as means (± S.D.). Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids

Amino Acid (μM/g)	**Artemia**	**Commercial**	**W-W**	**W-T**	**T-W**	**T-T**
Aspartic Acid	0.68 (± 0.02)	1.29 (± 0.30)	0.75 (± 0.06)	0.87 (± 0.02)	0.74 (± 0.02)	0.93 (± 0.05)
Glutamic Acid	1.38 (± 0.03)	1.74 (± 0.26)	1.57 (± 0.10)	1.53 (± 0.10)	1.18 (± 0.03)	1.36 (± 0.05)
Asparagine	0.35 (± 0.05)	0.15 (± 0.04)	0.15 (± 0.01)	0.11 (± 0.01)	0.12 (± 0.01)	0.11 (± 0.01)
Serine	1.23 (± 0.08)	0.57 (± 0.09)	0.42 (± 0.01)	0.39 (± 0.02)	0.45 (± 0.01)	0.39 (± 0.04)
Glutamine	0.54 (± 0.04)	0.40 (± 0.06)	0.35 (± 0.02)	0.40 (± 0.04)	0.31 (± 0.01)	0.31 (± 0.00)
Histidine	1.52 (± 0.12)	0.54 (± 0.11)	1.26 (± 0.22)	0.55 (± 0.03)	0.51 (± 0.03)	0.39 (± 0.00)
Glycine	2.32 (± 0.17)	0.76 (± 0.18)	1.97 (± 0.08)	1.04 (± 0.01)	1.30 (± 0.29)	1.04 (± 0.06)
Threonine	0.87 (± 0.03)	0.96 (± 0.22)	0.93 (± 0.09)	0.87 (± 0.07)	0.85 (± 0.08)	0.73 (± 0.06)
Arginine	0.17 (± 0.00)	0.22 (± 0.02)	0.38 (± 0.01)	0.30 (± 0.04)	0.36 (± 0.03)	0.22 (± 0.05)
Alanine	1.66 (± 0.08)	1.32 (± 0.26)	0.83 (± 0.10)	0.92 (± 0.07)	0.87 (± 0.03)	0.73 (± 0.06)
Tyrosine	0.44 (± 0.08)	0.40 (± 0.04)	0.56 (± 0.01)	0.47 (± 0.02)	0.60 (± 0.01)	0.28 (± 0.26)
Lysine	0.69 (± 0.12)	1.46 (± 0.26)	2.16 (± 0.06)	1.85 (± 0.31)	1.82 (± 0.40)	1.53 (± 0.62)
Methionine	0.91 (± 0.17)	0.74 (± 0.06)	1.07 (± 0.10)	1.15 (± 0.25)	1.19 (± 0.06)	1.14 (± 0.07)
Valine	1.10 (± 0.11)	1.45 (± 0.06)	1.90 (± 0.05)	1.56 (± 0.39)	0.80 (± 0.48)	1.17 (± 0.02)
Cysteine *Not detected	-	-	-	-	-	-

Tryptophan	0.02 (± 0.00)	0.03 (± 0.00)	0.02 (± 0.00)	0.03 (± 0.02)	0.04 (± 0.00)	0.06 (± 0.01)
Phenylalanine	0.09 (± 0.01)	0.14 (± 0.01)	0.12 (± 0.01)	0.10 (± 0.01)	0.08 (± 0.03)	0.12 (± 0.02)
Isoleucine	0.08 (± 0.01)	0.11 (± 0.01)	0.09 (± 0.01)	0.07 (± 0.04)	0.02 (± 0.00)	0.11 (± 0.02)
Leucine	0.16 (± 0.03)	0.25 (± 0.01)	0.22 (± 0.03)	0.19 (± 0.10)	0.17 (± 0.08)	0.24 (± 0.01)
Proline	0.53 (± 0.10)	0.49 (± 0.09)	0.37 (± 0.03)	0.22 (± 0.03)	0.10 (± 0.08)	0.20 (± 0.04)
IDAA	5.61 (± 0.30)	5.90 (± 0.68)	8.14 (± 0.16)	6.67 (± 0.57)	5.84 (± 0.92)	5.71 (± 0.86)
DAA	9.12 (± 0.41)	7.12 (± 1.22)	6.95 (± 0.14)	5.97 (± 0.29)	5.66 (± 0.37)	5.35 (± 0.25)
TFAA	14.73 (± 0.63)	13.02 (± 1.84)	15.09 (± 0.23)	12.64 (± 0.74)	11.50 (± 1.19)	11.06 (± 1.09)

W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes

Table 4.1. Diet formulations for Zebrafish larvae feeding trial.

Ingredients (%)	Unhydro	50% Hydro	100% Hydro
Unhydrolyzed Zebrafish meal	74.00	37.00	-
Hydrolysate Mix[a]	-	38.75	78.00
Starch	4.85	3.10	0.85
Krill Meal[b]	5.00	5.00	5.00
Fish oil[c]	4.00	4.00	4.00
Lecithin[d]	4.00	4.00	4.00
Mineral mix[e]	3.00	3.00	3.00
Vitamin mix[f]	3.00	3.00	3.00
$CaHPO_4$	1.00	1.00	1.00
Taurine	1.00	1.00	1.00
Choline chloride	0.10	0.10	0.10
Vitamin C[g]	0.05	0.05	0.05
Sum	100	100	100

[a] Equal mixture of 1, 2, and 3 h hydrolyzed Zebrafish meals
[b] Processed *Euphausia superba* (Florida Aqua Farms, Dade City, FL, USA).
[c] Cod liver oil (MP Biomedicals, Solon, OH, USA).
[d] Refined soy lecithin (MP Biomedicals, Solon, OH, USA).
[e] Bernhart-Tomarelli mineral mix with 5ppm selenium in a form of sodium selenite (Dyets, Bethlehem, PA, USA).
[f] Custom Vitamin Mixture (mg/kg diet) Thiamin HCl, 6.84; Riboflavin, 7.2; Pyridoxine HCl, 10.29; Niacin, 16.35; D-Calcium Pantothenate, 75.84; Folic Acid, 1.89; D-Biotin, 0.24; Vitamin B12 (0.1%), 30; Vitamin A Palmitate (500,000 IU/g), 14.49; Vitamin D3 (400,000 IU/g), 12.39; Vitamin E Acetate (500 IU/g), 198; Menadione Sodium Bisulfite, 3.54; Inositol, 750 (Dyets, Bethlehem, PA, USA).
[g] L-Ascorbyl-2-Polyphosphate (Argent Aquaculture, Redmond, WA, USA).

Table 4.2. Analyzed composition of formulated diets. Diets were analyzed in triplicates and the values are presented as mean (± S.D.)

Analyzed Composition (g/100g) Dry Matter	**Unhydro**	**50% Hydro**	**100% Hydro**
Crude Protein (N x 6.25)	48.14 (± 0.18)	48.19 (± 0.09)	48.26 (± 0.17)
Crude Lipids	10.04 (± 0.08)	10.07 (± 0.14)	10.06 (± 0.12)
Ash	17.92 (± 0.04)	17.69 (± 0.05)	17.49 (± 0.03)
Alanine	2.94 (± 0.03)	2.88 (± 0.02)	2.90 (± 0.02)
Arginine	2.81 (± 0.01)	2.67 (± 0.04)	2.64 (± 0.02)
Aspartic Acid	4.32 (± 0.03)	4.26 (± 0.03)	4.24 (± 0.01)
Cysteine	0.46 (± 0.00)	0.46 (± 0.00)	0.48 (± 0.00)
Glutamic Acid	6.32 (± 0.15)	6.16 (± 0.06)	6.20 (± 0.08)
Glycine	2.92 (± 0.03)	2.80 (± 0.03)	2.93 (± 0.03)
Histidine	1.20 (± 0.00)	1.17 (± 0.01)	1.11 (± 0.02)
Hydroxyproline	0.58 (± 0.01)	0.55 (± 0.02)	0.61 (± 0.00)
Isoleucine	2.24 (± 0.01)	2.23 (± 0.02)	2.17 (± 0.02)
Leucine	3.56 (± 0.02)	3.52 (± 0.03)	3.45 (± 0.02)
Lysine	3.77 (± 0.03)	3.72 (± 0.03)	3.72 (± 0.01)
Methionine	1.20 (± 0.01)	1.17 (± 0.01)	1.19 (± 0.00)
Phenylalanine	2.01 (± 0.02)	1.99 (± 0.02)	1.94 (± 0.04)
Proline	2.17 (± 0.02)	2.12 (± 0.02)	2.18 (± 0.03)
Serine	1.86 (± 0.07)	1.79 (± 0.04)	1.83 (± 0.05)
Taurine	1.64 (± 0.25)	1.58 (± 0.03)	1.42 (± 0.05)
Threonine	1.97 (± 0.01)	1.94 (± 0.02)	1.93 (± 0.01)
Tryptophan	0.45 (± 0.01)	0.42 (± 0.01)	0.42 (± 0.00)
Tyrosine	1.52 (± 0.00)	1.44 (± 0.10)	1.50 (± 0.01)
Valine	2.42 (± 0.01)	2.40 (± 0.03)	2.35 (± 0.02)
Sum	46.37 (± 0.37)	45.27 (± 0.40)	45.21 (± 0.21)

Table 4.3. Primers used for PepT1 analysis.

Gene	GenBank Accension #	Primers	Function	Reference
PepT1/*slc15a1*	AY300011	F: ATCATTGTGCTCATCGTGGC R: GGCGAAGATGATGCTCACAG	Di- and tripeptide absorption	Perera and Yúfera, 2016
ef1a	NM_131263	F: TTGAGAAGAAAATCGGTGGTGCTG R: GAACGGTGTGATTGAGGGAAATTC	Reference gene	Yossa et al. 2011

Table 4.4. Diet effect on growth and survival. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-Way ANOVA and a Tukey test with a p value < 0.05.

Group	Avg. Weight (mg)	Avg. Total Length (mm)	Survival (%)
LF	8.53^{c} (± 0.57)	9.77^{c} (± 0.32)	90.67^{b} (± 5.03)
Com	1.98^{b} (± 0.21)	4.99^{b} (± 0.21)	80.67^{ab} (± 3.06)
Unhydro	1.18^{ab} (± 0.10)	4.16^{a} (± 0.18)	76.33^{ab} (± 1.16)
50% Hydro	0.98^{a} (± 0.02)	4.16^{a} (± 0.32)	76.67^{ab} (± 4.16)
100% Hydro	0.96^{a} (± 0.20)	3.84^{a} (± 0.41)	65.00^{a} (± 19.70)

Table 4.5. Postprandial FAA composition in muscle. The values presented are postprandial levels, 2 h after feeding. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-way ANOVA and a Tukey test with a p value < 0.05. Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids.

Amino Acid (μM/g)	LF	Com	Unhydro	50% Hydro	100% Hydro
Aspartic Acid	1.58^{a} (± 0.06)	2.21^{ab} (± 0.70)	1.97^{ab} (± 0.04)	2.91^{b} (± 0.38)	2.17^{ab} (± 0.26)
Glutamic Acid	3.54^{b} (± 0.20)	4.11^{c} (± 0.04)	2.17^{a} (± 0.26)	1.86^{a} (± 0.15)	1.74^{a} (± 0.16)
Asparagine	0.87^{c} (± 0.13)	0.89^{c} (± 0.04)	0.42^{b} (± 0.09)	0.21^{a} (± 0.01)	0.27^{ab} (± 0.01)
Serine	1.67^{b} (± 0.17)	1.85^{b} (± 0.01)	1.00^{a} (± 0.13)	0.89^{a} (± 0.02)	0.78^{a} (± 0.03)
Glutamine	3.56^{c} (± 0.13)	3.21^{c} (± 0.05)	1.73^{b} (± 0.20)	1.37^{ab} (± 0.15)	1.25^{a} (± 0.09)
Histidine	3.22^{d} (± 0.18)	4.72^{e} (± 0.10)	2.22^{c} (± 0.26)	1.56^{b} (± 0.08)	1.00^{a} (± 0.06)
Glycine	3.10^{d} (± 0.15)	3.75^{e} (± 0.12)	2.29^{c} (± 0.22)	1.62^{b} (± 0.05)	0.96^{a} (± 0.09)
Threonine	1.48^{ab} (± 0.11)	4.11^{d} (± 0.11)	1.91^{c} (± 0.20)	1.82^{bc} (± 0.16)	1.13^{a} (± 0.09)
Arginine	0.82^{b} (± 0.14)	1.05^{c} (± 0.04)	0.51^{a} (± 0.04)	0.59^{a} (± 0.07)	0.46^{a} (± 0.03)
Alanine	4.94^{b} (± 0.43)	4.72^{b} (± 0.02)	1.98^{a} (± 0.19)	1.74^{a} (± 0.05)	1.44^{a} (± 0.08)
Tyrosine	6.45^{d} (± 0.15)	4.96^{c} (± 0.34)	2.75^{b} (± 0.32)	1.42^{a} (± 0.53)	0.71^{a} (± 0.04)
Lysine	1.95^{c} (± 0.26)	2.91^{d} (± 0.07)	1.13^{b} (± 0.10)	1.06^{ab} (± 0.08)	0.74^{a} (± 0.04)
Methionine	3.01^{c} (± 0.06)	3.61^{d} (± 0.07)	1.13^{b} (± 0.12)	1.10^{b} (± 0.14)	0.79^{a} (± 0.05)
Valine	1.15^{c} (± 0.12)	1.37^{d} (± 0.03)	0.61^{ab} (± 0.04)	0.67^{b} (± 0.08)	0.46^{a} (± 0.03)
Cysteine *Not detected	-	-	-	-	-
Tryptophan	0.19^{a} (± 0.08)	0.34^{c} (± 0.01)	0.21^{ab} (± 0.01)	0.32^{bc} (± 0.04)	0.31^{bc} (± 0.02)
Phenylalanine	0.42^{a} (± 0.06)	0.67^{b} (± 0.02)	0.38^{a} (± 0.05)	0.41^{a} (± 0.03)	0.32^{a} (± 0.02)
Isoleucine	0.73^{b} (± 0.13)	1.33^{c} (± 0.03)	0.66^{ab} (± 0.04)	0.73^{b} (± 0.08)	0.52^{a} (± 0.04)
Leucine	0.89^{ab} (± 0.13)	2.18^{c} (± 0.04)	1.06^{b} (± 0.05)	1.13^{b} (± 0.12)	0.77^{a} (± 0.05)
Proline	2.51^{d} (± 0.33)	1.64^{c} (± 0.07)	0.94^{b} (± 0.12)	0.72^{b} (± 0.08)	0.20^{a} (± 0.01)
IDAA	13.84^{c} (± 1.03)	22.28^{d} (± 0.38)	9.84^{b} (± 0.90)	9.40^{b} (± 0.77)	6.51^{a} (± 0.36)
DAA	28.22^{c} (± 1.63)	27.35^{c} (± 1.10)	15.26^{b} (± 1.52)	12.75^{b} (± 0.19)	9.51^{a} (± 0.75)
TFAA	42.06^{c} (± 2.66)	49.63^{d} (± 1.47)	25.10^{b} (± 2.42)	22.15^{b} (± 0.89)	16.02^{a} (± 1.11)

Table 4.6. Basal FAA composition in muscle. The values presented are postprandial levels, 24 h after feeding. Values are presented as means (± S.D.). Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids.

Amino Acid (μM/g)	LF	Com	Unhydro	50% Hydro	100% Hydro
Aspartic Acid	1.08 (± 0.22)	1.64 (± 0.07)	1.94 (± 0.03)	2.37 (± 0.03)	2.44 (± 0.21)
Glutamic Acid	2.28 (± 0.60)	2.11 (± 0.13)	1.29 (± 0.14)	1.82 (± 0.04)	2.07 (± 0.07)
Asparagine	0.52 (± 0.05)	0.40 (± 0.04)	0.15 (± 0.02)	0.11 (± 0.01)	0.29 (± 0.00)
Serine	0.43 (± 0.09)	0.59 (± 0.01)	0.35 (± 0.04)	0.67 (± 0.03)	0.64 (± 0.04)
Glutamine	2. 00 (± 0.57)	1.30 (± 0.08)	0.81 (± 0.09)	1.02 (± 0.01)	1.18 (± 0.05)
Histidine	2.46 (± 0.29)	3.17 (± 0.15)	1.10 (± 0.13)	1.49 (± 0.02)	1.48 (± 0.07)
Glycine	3.11 (± 0.21)	2.61 (± 0.14)	1.27 (± 0.17)	1.37 (± 0.02)	1.52 (± 0.07)
Threonine	0.26 (± 0.09)	2.06 (± 0.08)	0.88 (± 0.07)	1.15 (± 0.04)	0.93 (± 0.07)
Arginine	0.12 (± 0.05)	0.27 (± 0.01)	0.22 (± 0.02)	0.52 (± 0.03)	0.40 (± 0.02)
Alanine	2.51 (± 0.38)	1.93 (± 0.09)	1.07 (± 0.11)	2.03 (± 0.05)	1.75 (± 0.10)
Tyrosine	5.17 (± 1.27)	4.10 (± 0.37)	0.67 (± 0.08)	1.77 (± 0.45)	2.50 (± 0.14)
Lysine	0.35 (± 0.12)	0.74 (± 0.03)	0.39 (± 0.04)	0.82 (± 0.05)	0.63 (± 0.04)
Methionine	2.25 (± 0.53)	2.61 (± 0.08)	1.03 (± 0.11)	0.92 (± 0.02)	0.92 (± 0.01)
Valine	0.46 (± 0.08)	0.59 (± 0.04)	0.33 (± 0.03)	0.39 (± 0.01)	0.40 (± 0.02)
Cysteine *Not detected	-	-	-	-	-
Tryptophan	0.07 (± 0.04)	0.28 (± 0.01)	0.24 (± 0.03)	0.14 (± 0.00)	0.33 (± 0.01)
Phenylalanine	0.09 (± 0.02)	0.25 (± 0.01)	0.18 (± 0.03)	0.27 (± 0.02)	0.26 (± 0.02)
Isoleucine	0.11 (± 0.02)	0.23 (± 0.01)	0.19 (± 0.02)	0.33 (± 0.02)	0.30 (± 0.02)
Leucine	0.20 (± 0.05)	0.40 (± 0.02)	0.34 (± 0.03)	0.63 (± 0.04)	0.48 (± 0.03)
Proline	0.16 (± 0.21)	0.17 (± 0.02)	0.01 (± 0.00)	0.16 (± 0.00)	0.06 (± 0.01)
IDAA	6.39 (± 1.25)	10.60 (± 0.41)	4.90 (± 0.49)	6.66 (± 0.10)	6.14 (± 0.29)
DAA	17.25 (± 3.41)	14.84 (± 0.74)	7.56 (± 0.66)	11.32 (± 0.53)	12.45 (± 0.65)
TFAA	23.63 (± 4.63)	25.44 (± 1.15)	12.46 (± 1.15)	17.98 (± 0.63)	18.59 (± 0.93)

Table 5.1. Diet formulations for feeding trial.

Ingredients g/100g	**CG**	**FAA50**	**(-) Lys**	**(-) Met**	**(-) Thr**	**Def**
Casein	40.0	20.0	20.0	20.0	20.0	20.0
Gelatin	8.0	4.0	4.0	4.0	4.0	4.0
FAA mix	-	24.0	24.0	24.0	24.0	24.0
Dextrin	28.1	28.1	28.1	28.1	28.1	28.1
Fish Oil	10.0	10.0	10.0	10.0	10.0	10.0
Lecithin	4.7	4.7	4.7	4.7	4.7	4.7
Vitamin mix	2.5	2.5	2.5	2.5	2.5	2.5
Mineral mix	2.5	2.5	2.5	2.5	2.5	2.5
$CaHPO_4$	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin C	0.1	0.1	0.1	0.1	0.1	0.1
Choline chloride	0.1	0.1	0.1	0.1	0.1	0.1
Taurine	1.0	1.0	1.0	1.0	1.0	1.0
CMC	1.0	1.0	1.0	1.0	1.0	1.0

Table 5.2. Composition of FAA mix added to diets.

AA (g/100g	**FAA50**	**(-) Lys**	**(-) Met**	**(-) Thr**	**Def**
Arginine	4.26	4.26	4.26	4.26	4.26
Valine	5.72	5.72	5.72	5.72	5.72
Histidine	2.44	2.44	2.44	2.44	2.44
Leucine	7.97	7.97	7.97	7.97	7.97
Glycine	4.84	11.59	7.20	8.50	17.61
Isoleucine	4.45	4.45	4.45	4.45	4.45
Methionine	2.36	2.36	-	2.36	-
Phenylalanine	4.51	4.51	4.51	4.51	4.51
Alanine	4.00	4.00	4.00	4.00	4.00
Tyrosine	4.61	4.61	4.61	4.61	4.61
Lysine	6.75	-	6.75	6.75	-
Tryptophan	1.09	1.09	1.09	1.09	1.09
Threonine	3.66	3.66	3.66	-	-
Cysteine	0.31	0.31	0.31	0.31	0.31
Glutamic Acid	19.31	19.31	19.31	19.31	19.31
Hydroxyproline	2.04	2.04	2.04	2.04	2.04
Proline	10.76	10.76	10.76	10.76	10.76
Serine	4.34	4.34	4.34	4.34	4.34
Aspartic Acid	6.58	6.58	6.58	6.58	6.58

Table 5.3**.** Analyzed composition of formulated diets. Diets were analyzed in triplicates and the values are presented as mean (± S.D.).

Analyzed Composition (g/100g) Dry Matter	**CG**	**FAA50**	**(-) Lys**	**(-) Met**	**(-) Thr**	**Def**
Crude Protein (N x 6.25)	48.32 (± 0.11)	48.28 (± 0.11)	48.29 (± 0.20)	48.28 (± 0.15)	48.32 (± 0.21)	48.26 (± 0.07)
Crude Lipids	14.17 (± 0.08)	14.15 (± 0.05)	14.19 (± 0.06)	14.23 (± 0.11)	14.17 (± 0.03)	14.20 (± 0.07)
Ash	5.67 (± 0.02)	6.33 (± 0.02)	6.24 (± 0.06)	6.14 (± 0.04)	6.16 (± 0.02)	6.16 (± 0.01)
Alanine	1.79 (± 0.01)	1.46 (± 0.03)	1.84 (± 0.02)	1.87 (± 0.12)	1.89 (± 0.13)	1.86 (± 0.06)
Arginine	1.91 (± 0.01)	1.97 (± 0.03)	1.99 (± 0.01)	1.94 (± 0.02)	1.94 (± 0.04)	2.01 (± 0.00)
Aspartic Acid	2.97 (± 0.03)	3.03 (± 0.02)	3.09 (± 0.07)	3.12 (± 0.07)	2.99 (± 0.02)	3.12 (± 0.01)
Cysteine	0.14 (± 0.00)	0.14 (± 0.00)	0.14 (± 0.00)	0.16 (± 0.00)	0.15 (± 0.00)	0.15 (± 0.00)
Glutamic Acid	8.67 (± 0.06)	7.99 (± 0.06)	8.10 (± 0.02)	8.00 (± 0.11)	8.02 (± 0.09)	8.07 (± 0.01)
Glycine	2.36 (± 0.05)	2.33 (± 0.15)	3.93 (± 0.33)	2.88 (± 0.05)	3.30 (± 0.03)	5.19 (± 0.07)
Histidine	1.12 (± 0.01)	1.13 (± 0.01)	1.19 (± 0.00)	1.10 (± 0.02)	1.14 (± 0.01)	1.13 (± 0.01)
Hydroxyproline	0.82 (± 0.01)	0.85 (± 0.01)	0.83 (± 0.01)	0.84 (± 0.03)	0.89 (± 0.02)	0.89 (± 0.08)
Isoleucine	2.16 (± 0.01)	2.11 (± 0.02)	2.08 (± 0.01)	2.10 (± 0.07)	2.10 (± 0.03)	2.08 (± 0.03)
Leucine	3.75 (± 0.01)	3.86 (± 0.02)	3.81 (± 0.01)	3.81 (± 0.04)	3.77 (± 0.06)	3.81 (± 0.03)
Lysine	3.15 (± 0.01)	3.24 (± 0.04)	1.77 (± 0.00)	3.24 (± 0.07)	3.32 (± 0.08)	1.72 (± 0.01)
Methionine	1.08 (± 0.01)	1.03 (± 0.03)	1.11 (± 0.02)	0.59 (± 0.01)	1.07 (± 0.05)	0.58 (± 0.01)
Phenylalanine	2.08 (± 0.00)	2.25 (± 0.10)	2.15 (± 0.01)	2.20 (± 0.03)	2.04 (± 0.04)	2.15 (± 0.02)
Proline	4.81 (± 0.02)	4.92 (± 0.05)	4.93 (± 0.05)	4.92 (± 0.03)	4.91 (± 0.08)	4.96 (± 0.04)
Serine	1.94 (± 0.01)	1.90 (± 0.18)	1.78 (± 0.09)	1.69 (± 0.04)	1.85 (± 0.12)	1.68 (± 0.08)
Taurine	0.92 (± 0.03)	1.04 (± 0.10)	1.01 (± 0.03)	1.14 (± 0.08)	1.15 (± 0.10)	1.16 (± 0.04)
Threonine	1.64 (± 0.01)	1.64 (± 0.02)	1.64 (± 0.00)	1.65 (± 0.02)	0.89 (± 0.01)	0.87 (± 0.00)

Tryptophan	0.50 (± 0.00)	0.51 (± 0.01)	0.53 (± 0.02)	0.53 (± 0.01)	0.50 (± 0.03)	0.52 (± 0.01)
Tyrosine	2.05 (± 0.02)	2.62 (± 0.08)	2.08 (± 0.03)	1.97 (± 0.09)	1.96 (± 0.13)	2.07 (± 0.01)
Valine	2.65 (± 0.01)	2.66 (± 0.02)	2.71 (± 0.03)	2.67 (± 0.05)	2.65 (± 0.01)	2.66 (± 0.02)
Sum	46.42 (± 0.09)	46.88 (± 0.20)	46.71 (± 0.19)	46.41 (± 0.25)	46.54 (± 0.08)	46.67 (± 0.08)

Table 5.4. Primers utilized for RT-PCR analysis.

Gene	GenBank Accension #	Primers	Function
Ghrelin	AM055940.1	F: GTGTCTCGAGTCTGTGAGCG R: CAGCTTCTCTTCTGCCCACT	Orexigenic Hormone
Leptin	BN000830.1	F: TGTTGACCAGATACGCCGAG R: GTCCAGCGCTTTCCCATTTG	Anorexigenic Hormone
CCK	XM_001346104.6	F: GTTCAGTCTAATGTCGGCTCC R: TAGTTCGGTTAGGCTGCTGC	Anorexigenic Hormone
Orexin	NM_001077392	F: CTACGAGATGCTGTGCCGAG R: GAGTGAGAATCCCGACAGCG	Orexigenic Hormone
NPY	BC162071.1	F: TGGGGACTCTCACAGAAGGG R: AATACTTGGCGAGCTCCTCC	Orexigenic Hormone
gpcr6a	ZDB-GENE-041217-22	F: CCGAAACGGAGGGAATTTGC R: TATCACTGGCAACCCACACC	Sensing of Luminal AA
casr	ZDB-GENE-050119-8	F: AAGAGATGGTGAGACGAAACAT R: CATGACGTCAAGATACTCTGGT	Calcium-sensing Receptor
tas1r2	NM_001039831	F: ACGTCTAAACCTTGATGCAAAC R: CACTCGCAATCCATACTTTGTC	Sensing of L-Amino Acids
ef1a	NM_131263	F: TTGAGAAGAAAATCGGTGGTGCTG R: GAACGGTGTGATTGAGGGAAATTC	Reference gene

Table 5.5. Growth and feeding parameters. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-Way ANOVA and a Tukey test with a p value < 0.05.

Group	Avg. Weight (mg)	Avg. Weight Gain (%)	Avg. Feed Intake (mg/fish/day)	Feeding Efficiency	Survival (%)
CG	39.48^{b} (± 4.51)	679.29^{b} (± 89.01)	54.81^{c} (± 2.09)	0.63^{b} (± 0.06)	97.78 (± 1.92)
FAA50	28.93^{a} (± 1.84)	470.98^{a} (± 36.41)	45.04^{a} (± 1.99)	0.53^{ab} (± 0.02)	97.78 (± 1.92)
(-) Lys	28.24^{a} (± 2.94)	457.39^{a} (± 57.97)	48.00^{ab} (± 1.22)	0.48^{a} (± 0.06)	97.78 (± 1.92)
(-) Met	30.18^{a} (± 1.84)	495.68^{a} (± 36.28)	48.04^{ab} (± 0.93)	0.52^{ab} (± 0.05)	95.56 (± 1.92)
(-) Thr	30.80^{a} (± 2.18)	507.87^{a} (± 43.11)	46.93^{ab} (± 1.52)	0.55^{ab} (± 0.03)	97.78 (± 1.92)
Def	29.50^{a} (± 1.91)	482.22^{a} (± 37.72)	49.26^{b} (± 1.02)	0.49^{a} (± 0.04)	98.89 (± 1.92)

Table 5.6. Postprandial FAA composition in muscle. The values presented are postprandial levels, 2 h after feeding. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-way ANOVA and a Tukey test with a p value < 0.05. Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids

Amino Acid (μM/g)	**CG**	**FAA50**	**(-) Lys**	**(-) Met**	**(-) Thr**	**Def**
Aspartic Acid	1.45 (± 0.33)	1.17 (± 0.12)	1.05 (± 0.18)	1.41 (± 0.18)	1.21 (± 0.00)	1.18 (± 0.30)
Glutamic Acid	1.70^{ab} (± 0.13)	2.12^{c} (± 0.10)	1.59^{a} (± 0.07)	2.02^{bc} (± 0.13)	1.86^{ac} (± 0.15)	1.98^{bc} (± 0.11)
Asparagine	1.02^{d} (± 0.03)	0.83^{c} (± 0.03)	0.69^{ab} (± 0.10)	0.75^{ac} (± 0.04)	0.83^{bc} (± 0.03)	0.66^{a} (± 0.03)
Serine	0.64^{a} (± 0.04)	0.99^{ab} (± 0.13)	1.21^{b} (± 0.16)	0.69^{a} (± 0.05)	0.66^{a} (± 0.14)	1.35^{b} (± 0.27)
Glutamine	2.10^{ab} (± 0.18)	2.44^{b} (± 0.10)	1.85^{a} (± 0.19)	2.28^{ab} (± 0.05)	2.03^{ab} (± 0.14)	2.10^{ab} (± 0.29)
Histidine	9.22^{ab} (± 0.55)	10.52^{b} (± 0.60)	10.65^{b} (± 0.60)	10.58^{b} (± 0.30)	8.29^{a} (± 1.09)	8.64^{a} (± 0.42)
Glycine	2.10^{a} (± 0.38)	3.15^{b} (± 0.20)	3.60^{bc} (± 0.11)	3.86^{c} (± 0.18)	3.73^{bc} (± 0.21)	5.28^{d} (± 0.12)
Threonine	4.50^{c} (± 0.71)	2.24^{ab} (± 0.22)	4.19^{c} (± 0.46)	3.26^{bc} (± 0.69)	1.89^{a} (± 0.13)	1.71^{a} (± 0.24)
Arginine	0.36 (± 0.05)	0.44 (± 0.14)	0.50 (± 0.08)	0.42 (± 0.11)	0.40 (± 0.10)	0.37 (± 0.15)
Alanine	1.57^{a} (± 0.11)	3.34^{bc} (± 0.40)	2.65^{b} (± 0.34)	2.78^{bc} (± 0.16)	2.89^{bc} (± 0.27)	3.46^{c} (± 0.16)
Tyrosine	0.06^{a} (± 0.01)	0.09^{ab} (± 0.03)	0.12^{ab} (± 0.01)	0.14^{b} (± 0.00)	0.11^{ab} (± 0.03)	0.14^{b} (± 0.05)
Lysine	2.22^{b} (± 0.32)	1.72^{ab} (± 0.22)	1.52^{ab} (± 0.20)	1.77^{ab} (± 0.30)	1.43^{a} (± 0.27)	1.17^{a} (± 0.20)
Methionine	6.42^{b} (± 0.34)	6.13^{b} (± 0.25)	6.25^{b} (± 0.50)	5.57^{ab} (± 0.37)	6.28^{b} (± 0.22)	4.83^{a} (± 0.30)
Valine	1.58^{ab} (± 0.12)	1.65^{ab} (± 0.06)	1.69^{b} (± 0.08)	1.37^{ab} (± 0.15)	1.52^{ab} (± 0.09)	1.35^{a} (± 0.16)
Cysteine *Not detected	-	-	-	-	-	-
Tryptophan	0.03 (± 0.00)	0.03 (± 0.00)	0.03 (± 0.00)	0.03 (± 0.00)	0.03 (± 0.00)	0.02 (± 0.01)
Phenylalanine	0.35^{a} (± 0.03)	0.51^{ab} (± 0.09)	0.71^{ab} (± 0.06)	0.96^{b} (± 0.45)	0.42^{ab} (± 0.14)	0.49^{ab} (± 0.17)
Isoleucine	0.35^{a} (± 0.02)	0.57^{b} (± 0.10)	0.45^{ab} (± 0.14)	0.36^{ab} (± 0.03)	0.36^{ab} (± 0.08)	0.33^{a} (± 0.05)
Leucine	0.69 (± 0.07)	1.14 (± 0.27)	1.08 (± 0.20)	0.71 (± 0.13)	0.92 (± 0.20)	0.85 (± 0.25)

Proline	8.29[b] (± 0.35)	8.23[ab] (± 2.07)	10.84[b] (± 2.28)	8.51[b] (± 2.30)	3.97[a] (± 0.81)	8.83[b] (± 0.72)
IDAA	25.71[c] (± 1.66)	24.95[bc] (± 1.87)	27.07[c] (± 0.80)	25.02[bc] (± 1.78)	21.54[ab] (± 1.65)	19.76[a] (± 0.97)
DAA	18.93[ab] (± 0.74)	22.37[bc] (± 2.51)	23.61[bc] (± 2.44)	22.45[bc] (± 2.03)	17.29[a] (± 1.36)	24.97[c] (± 1.00)
TFAA	44.64[ab] (± 2.28)	47.31[b] (± 3.63)	50.69[b] (± 3.24)	47.47[b] (± 3.03)	38.83[a] (± 2.90)	44.73[ab] (± 1.79)

Table 5.7. Basal FAA composition in muscle. The values presented are basal levels, 24 h after feeding. Values are presented as means (± S.D.). Superscript letters indicate statistical significance between groups. The significance was determined using a One-way ANOVA and a Tukey test with a p value < 0.05. Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids

Amino Acid (μM/g)	CG	FAA50	(-) Lys	(-) Met	(-) Thr	Def
Aspartic Acid	1.22 (± 0.07)	1.54 (± 0.16)	1.72 (± 0.08)	1.82 (± 0.05)	1.73 (± 0.10)	1.52 (± 0.14)
Glutamic Acid	2.15 (± 0.05)	2.21 (± 0.13)	2.17 (± 0.21)	2.10 (± 0.12)	2.25 (± 0.20)	2.37 (± 0.17)
Asparagine	0.90 (± 0.09)	0.62 (± 0.06)	0.86 (± 0.03)	0.79 (± 0.01)	0.81 (± 0.03)	0.95 (± 0.07)
Serine	0.28 (± 0.01)	0.25 (± 0.04)	0.37 (± 0.02)	0.29 (± 0.01)	0.39 (± 0.01)	0.58 (± 0.06)
Glutamine	2.13 (± 0.14)	2.55 (± 0.16)	2.26 (± 0.17)	2.10 (± 0.11)	2.17 (± 0.08)	2.46 (± 0.11)
Histidine	10.27 (± 0.91)	10.88 (± 1.00)	9.29 (± 0.73)	10.57 (± 0.64)	9.44 (± 0.57)	10.47 (± 0.30)
Glycine	3.77 (± 0.13)	2.27 (± 0.35)	3.46 (± 0.39)	4.25 (± 0.18)	3.58 (± 0.22)	5.94 (± 0.79)
Threonine	4.16 (± 0.78)	2.19 (± 0.25)	2.48 (± 0.44)	3.37 (± 0.25)	1.19 (± 0.18)	2.14 (± 0.31)
Arginine	0.08 (± 0.01)	0.05 (± 0.01)	0.04 (± 0.01)	0.05 (± 0.00)	0.10 (± 0.00)	0.06 (± 0.01)
Alanine	2.76 (± 0.24)	3.19 (± 0.19)	3.19 (± 0.19)	3.48 (± 0.27)	3.22 (± 0.32)	4.14 (± 0.30)
Tyrosine	0.12 (± 0.03)	0.09 (± 0.01)	0.09 (± 0.04)	0.13 (± 0.06)	0.07 (± 0.02)	0.14 (± 0.04)
Lysine	1.46 (± 0.27)	1.03 (± 0.21)	0.46 (± 0.04)	0.76 (± 0.02)	0.49 (± 0.05)	1.01 (± 0.59)
Methionine	4.83 (± 0.10)	6.41 (± 0.37)	6.72 (± 0.57)	5.18 (± 0.62)	6.67 (± 1.02)	5.38 (± 0.09)
Valine	0.95 (± 0.01)	1.08 (± 0.04)	1.13 (± 0.06)	0.95 (± 0.07)	1.14 (± 0.13)	1.07 (± 0.02)
Cysteine	0.01 (± 0.01)	0.01 (± 0.01)	0.24 (± 0.07)	0.02 (± 0.01)	0.01 (± 0.00)	0.12 (± 0.11)
Tryptophan	0.02 (± 0.00)	0.02 (± 0.00)	0.06 (± 0.02)	0.02 (± 0.00)	0.02 (± 0.00)	0.04 (± 0.03)
Phenylalanine	0.09 (± 0.01)	0.07 (± 0.01)	0.17 (± 0.02)	0.07 (± 0.01)	0.09 (± 0.01)	0.14 (± 0.05)
Isoleucine	0.09 (± 0.01)	0.07 (± 0.01)	0.29 (± 0.06)	0.08 (± 0.01)	0.09 (± 0.01)	0.23 (± 0.12)
Leucine	0.14 (± 0.02)	0.12 (± 0.01)	0.16 (± 0.01)	0.13 (± 0.01)	0.14 (± 0.02)	0.24 (± 0.04)

Proline	8.69 (± 0.98)	10.13 (± 0.42)	4.18 (± 0.82)	6.67 (± 0.37)	4.06 (± 0.43)	6.18 (± 0.15)
IDAA	22.09 (± 0.22)	21.93 (± 1.41)	20.81 (± 0.66)	21.17 (± 0.42)	19.37 (± 0.85)	20.78 (± 0.54)
DAA	22.01 (± 1.10)	22.86 (± 0.63)	18.30 (± 1.12)	21.63 (± 0.90)	18.28 (± 0.76)	24.28 (± 0.99)
TFAA	44.10 (± 1.02)	44.79 (± 1.00)	39.11 (± 1.26)	42.80 (± 0.83)	37.66 (± 0.58)	45.07 (± 1.37)

Table 6.1. Dietary formulations.

Ingredients (%)	**CG**	**FAA50**
Casein	40.0	20.0
Gelatin	8.0	4.0
FAA mix	-	24.0
Dextrin	31.3	31.3
Fish Oil[a]	10.0	10.0
Vitamin mix[b]	2.0	2.0
Mineral mix[c]	2.5	2.5
$CaHPO_4$	2.0	2.0
Vitamin C[d]	0.1	0.1
Choline chloride	0.1	0.1
Taurine	1.0	1.0
Carboxymethylcellulose	3.0	3.0

[a]Cod liver oil (MP Biomedicals, Solon, OH, USA).

[b] Custom Vitamin Mixture (mg/kg diet) Thiamin HCl, 6.84; Riboflavin, 7.2; Pyridoxine HCl, 10.29; Niacin, 16.35; D-Calcium Pantothenate, 75.84; Folic Acid, 1.89; D-Biotin, 0.24; Vitamin B12 (0.1%), 30; Vitamin A Palmitate (500,000 IU/g), 14.49; Vitamin D3 (400,000 IU/g), 12.39; Vitamin E Acetate (500 IU/g), 198; Menadione Sodium Bisulfite, 3.54; Inositol, 750 (Dyets, Bethlehem, PA, USA).

[c] Bernhart-Tomarelli mineral mix with 5 ppm selenium in a form of sodium selenite (Dyets, Bethlehem, PA, USA).

[d] L-Ascorbyl-2-Polyphosphate (Argent Aquaculture, Redmond, WA, USA).

Table 6.2. Composition of the FAA mix.

Amino Acid	**g/100g FAA mix**
Arginine	3.94
Valine	5.33
Histidine	2.43
Leucine	7.76
Glycine	6.33
Isoleucine	4.28
Methionine	2.36
Phenylalanine	4.32
Alanine	3.60
Tyrosine	4.37
Lysine HCl	6.81
Tryptophan	1.07
Threonine	3.93
Cysteine $HCLH_2O$	0.37
Glutamic acid	8.61
Hydroxyproline	1.94
Proline	11.98
Serine	4.75
Aspartic acid	3.66
Asparagine	2.35
Glutamine	9.82

Table 6.3. Analyzed composition of formulated diets.

Analyzed Composition (g/100g) Dry Matter	**CG**	**FAA50**
Crude Protein (N x 6.25)	49.39	49.77
Crude Lipids	10.09	10.23
Ash	6.50	6.38
Alanine	1.87	1.88
Arginine	1.81	1.89
Aspartic Acid	2.96	2.95
Cysteine	0.17	0.19
Glutamic Acid	8.50	8.80
Glycine	2.65	2.88
Histidine	1.01	1.09
Hydroxyproline	0.89	0.44
Isoleucine	2.05	2.12
Leucine	3.54	3.75
Lysine	3.13	2.99
Methionine	1.13	1.09
Phenylalanine	1.95	2.13
Proline	4.76	5.55
Serine	1.97	1.74
Taurine	1.28	1.61
Threonine	1.72	1.72
Tryptophan	0.46	0.45
Tyrosine	1.75	1.88
Valine	2.48	2.60
Sum AA	46.12	47.82

Table 6.4. Correlation coefficients between dietary indispensable amino acids (IDAA) and free IDAA patterns of plasma in Largemouth Bass (LMB), Walleye, and Zebrafish. IDAA = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Asterisks indicate significant correlations ($p < 0.05$).

	Plasma					
	LMB		Walleye		Zebrafish	
Time (h)	CG	FAA50	CG	FAA50	CG	FAA50
0	0.429	0.459	0.503	0.347	0.433	0.427
0.5	0.575	0.580	0.760*	0.192	0.708*	0.050
1	0.469	0.590	0.877*	0.689*	0.461	0.193
2	0.757*	0.696*	0.834*	0.810*	0.235	0.467
3	0.639*	0.579	0.851*	0.830*	0.322	-0.211
6	0.411	0.487	0.803*	0.713*	0.014	-0.148
12	0.518	0.527	0.777*	0.745*	0.069	-0.209

Table 6.5. Correlation coefficients between dietary indispensable amino acids (IDAA) and free IDAA patterns of liver in Largemouth Bass (LMB), Walleye, and Zebrafish. IDAA = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Asterisks indicate significant correlations ($p < 0.05$).

	Liver					
	LMB		Walleye		Zebrafish	
Time (h)	CG	FAA50	CG	FAA50	CG	FAA50
0	-0.192	-0.214	-0.214	-0.220	0.507	-0.120
0.5	-0.174	-0.175	-0.125	0.110	-0.106	-0.011
1	-0.174	-0.170	-0.037	0.898*	0.062	0.177
2	-0.096	-0.055	-0.037	0.335	0.041	-0.110
3	-0.053	-0.100	-0.082	-0.023	-0.031	-0.186
6	-0.078	-0.073	-0.123	-0.051	-0.097	-0.191
12	-0.117	-0.178	-0.154	-0.156	-0.149	-0.233

Table 6.6. Correlation coefficients between dietary indispensable amino acids (IDAA) and free IDAA patterns of muscle in Largemouth Bass (LMB), Walleye, and Zebrafish. IDAA = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Asterisks indicate significant correlations ($p < 0.05$).

	Muscle					
	LMB		Walleye		Zebrafish	
Time (h)	CG	FAA50	CG	FAA50	CG	FAA50
0	-0.335	-0.395	-0.273	-0.326	-0.351	-0.352
0.5	-0.343	-0.380	-0.253	-0.240	-0.371	-0.389
1	-0.299	-0.400	-0.210	-0.214	-0.370	-0.379
2	-0.334	-0.318	-0.281	-0.245	-0.371	-0.377
3	-0.354	-0.334	-0.320	-0.255	-0.344	-0.319
6	-0.318	-0.314	-0.267	-0.218	-0.344	-0.350
12	-0.317	-0.353	-0.243	-0.263	-0.362	-0.383

FIGURES

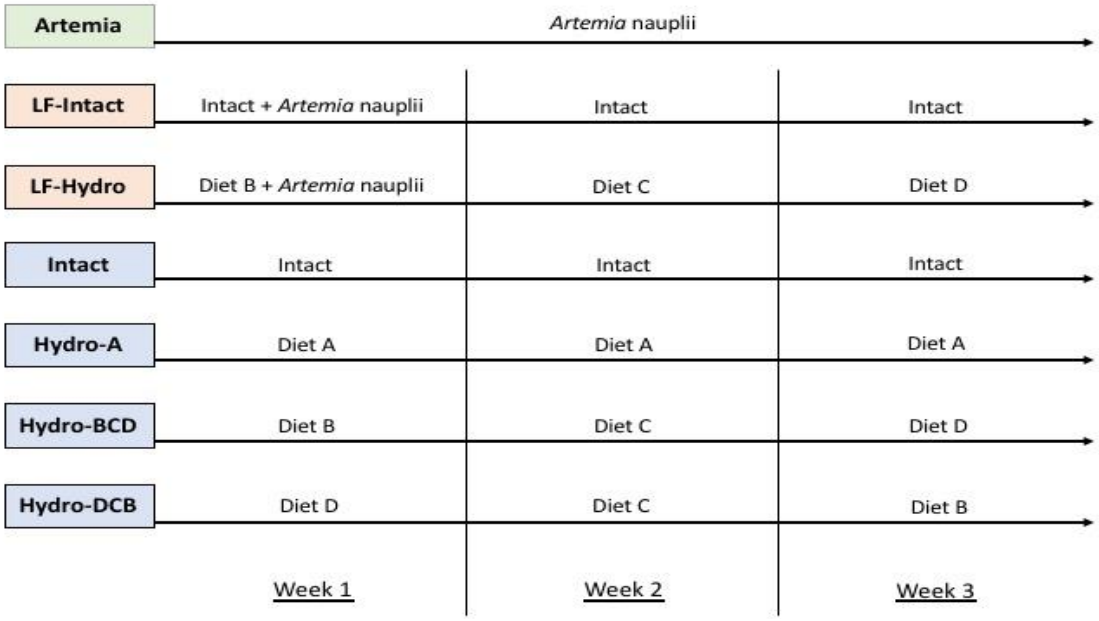

Figure 2.1. Feeding regimens for experimental groups. All diets were fed in excess during each feeding. The LF-Intact and LF-Hydro groups were fed decreasing amounts of Artemia starting on day 3 to wean them completely onto dry feed starting with week 2. The switch in diets commenced immediately after the sampling and measuring conducted at the end of each week.

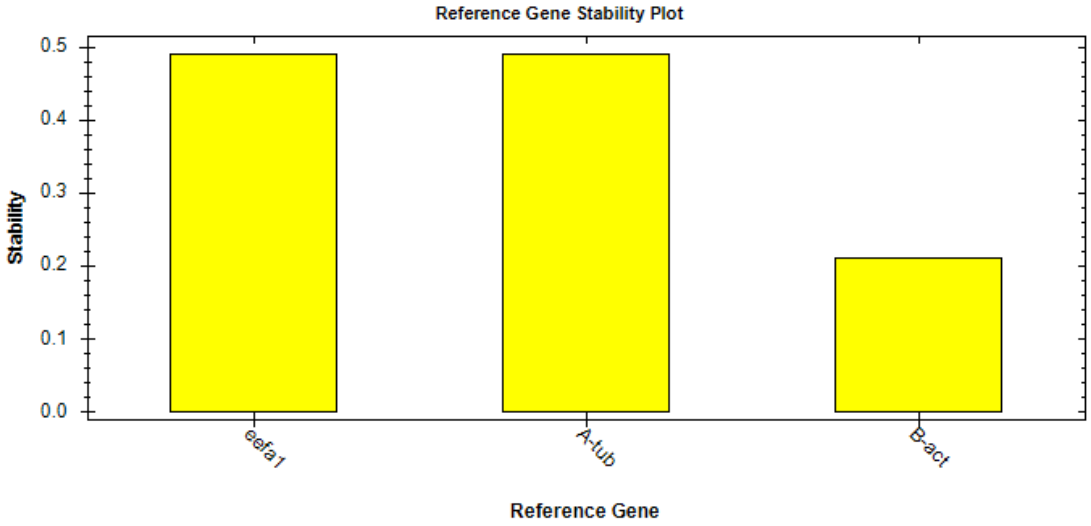

Figure 2.2. Results of stability analysis of the housekeeping genes *eEF1a1*, *α-tubulin,* and *β-actin*.

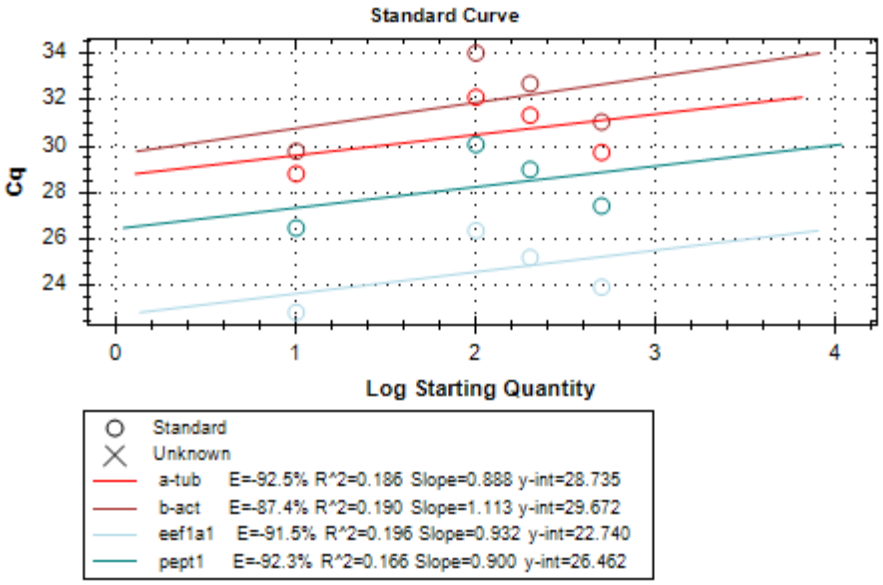

Figure 2.3. Results of amplification efficiency of each pair of primers for the target and housekeeping genes *eEF1a1*, *α-tubulin*, *β-actin*, and *PepT1*.

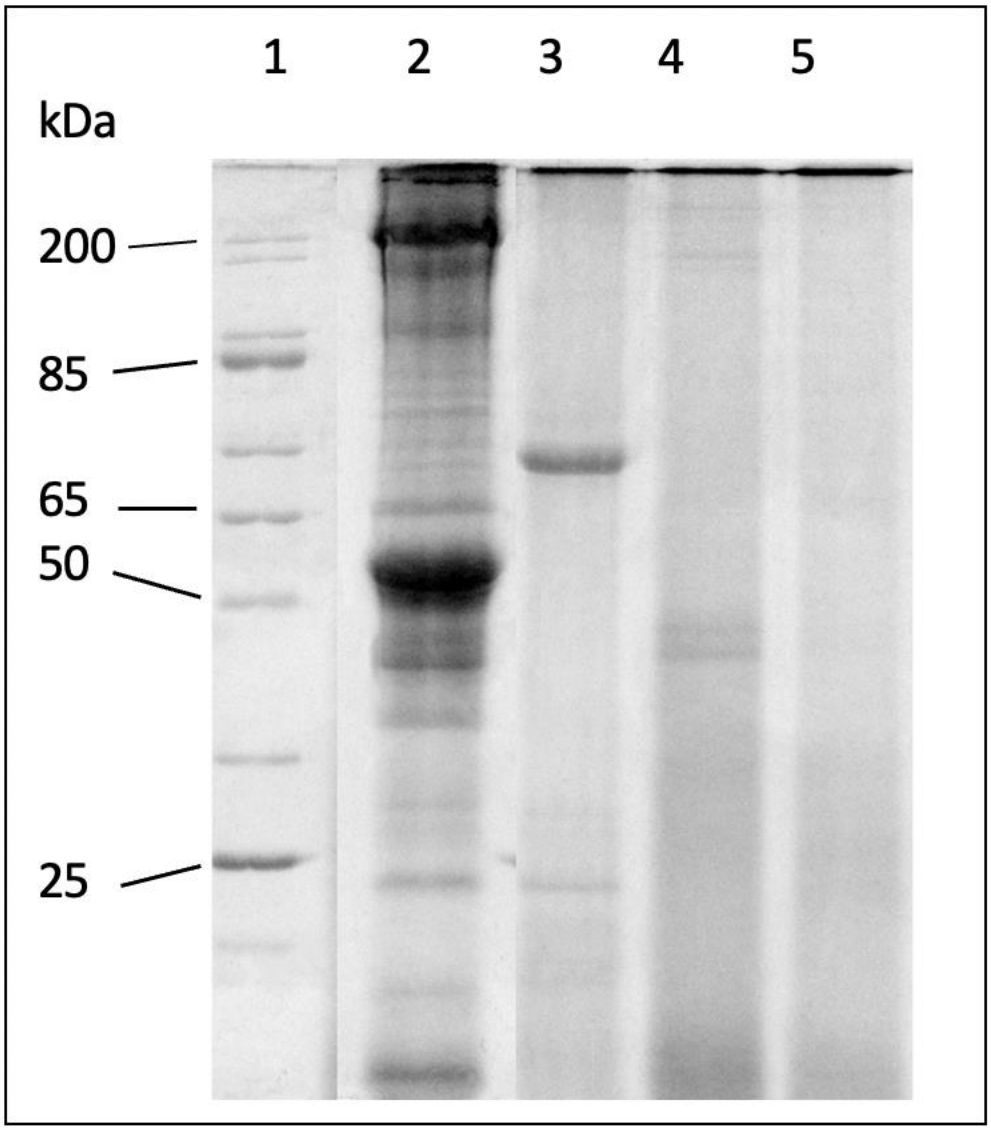

Figure 2.4. Representative reducing 12.5% T SDS-PAGE of Largemouth Bass muscle hydrolysis over time. Lanes 1) broad range molecular wgt standard (200 – 10 kDa); 2) intact fish muscle; 3) 1.5 h hydrolysis; 4) 3 h hydrolysis; 5) 6 h hydrolysis.

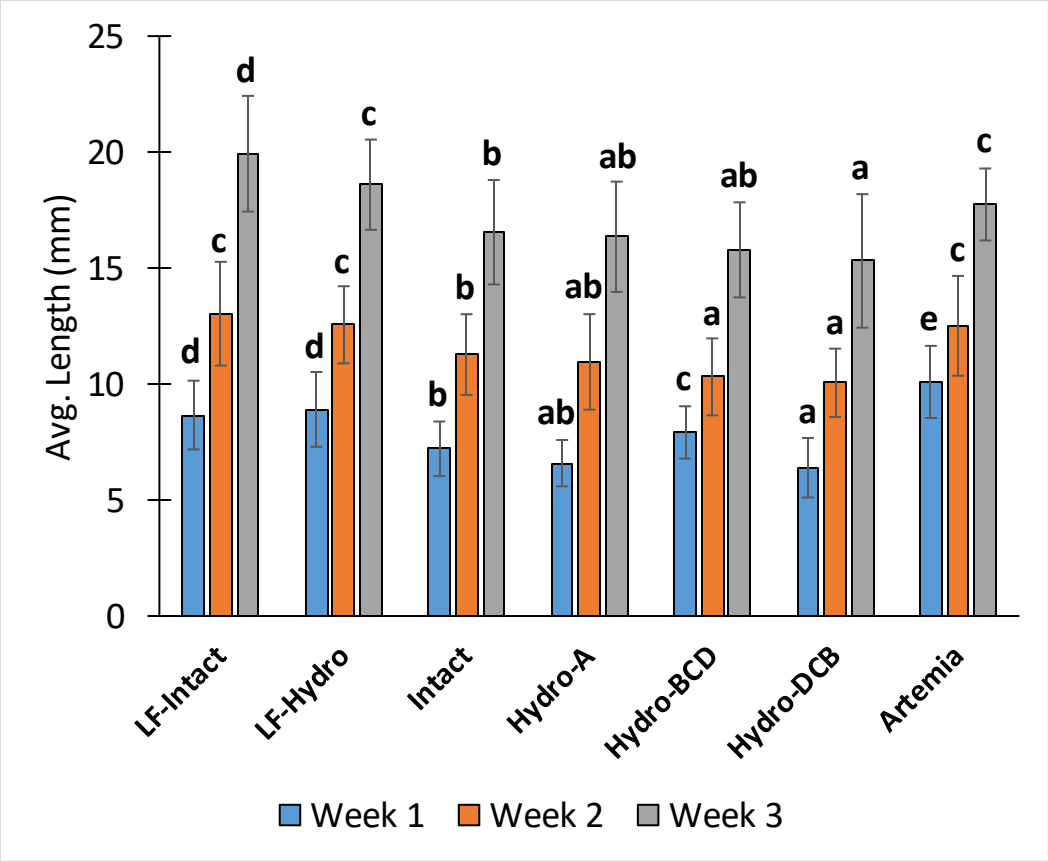

Figure 2.5. Diet effect on the average total length at the end of each week. Values are presented as means and error bars represent standard deviation for total length. Letters indicate statistical significance between groups. The significance was determined using a One-Way ANOVA and a Tukey test with a p value < 0.05.

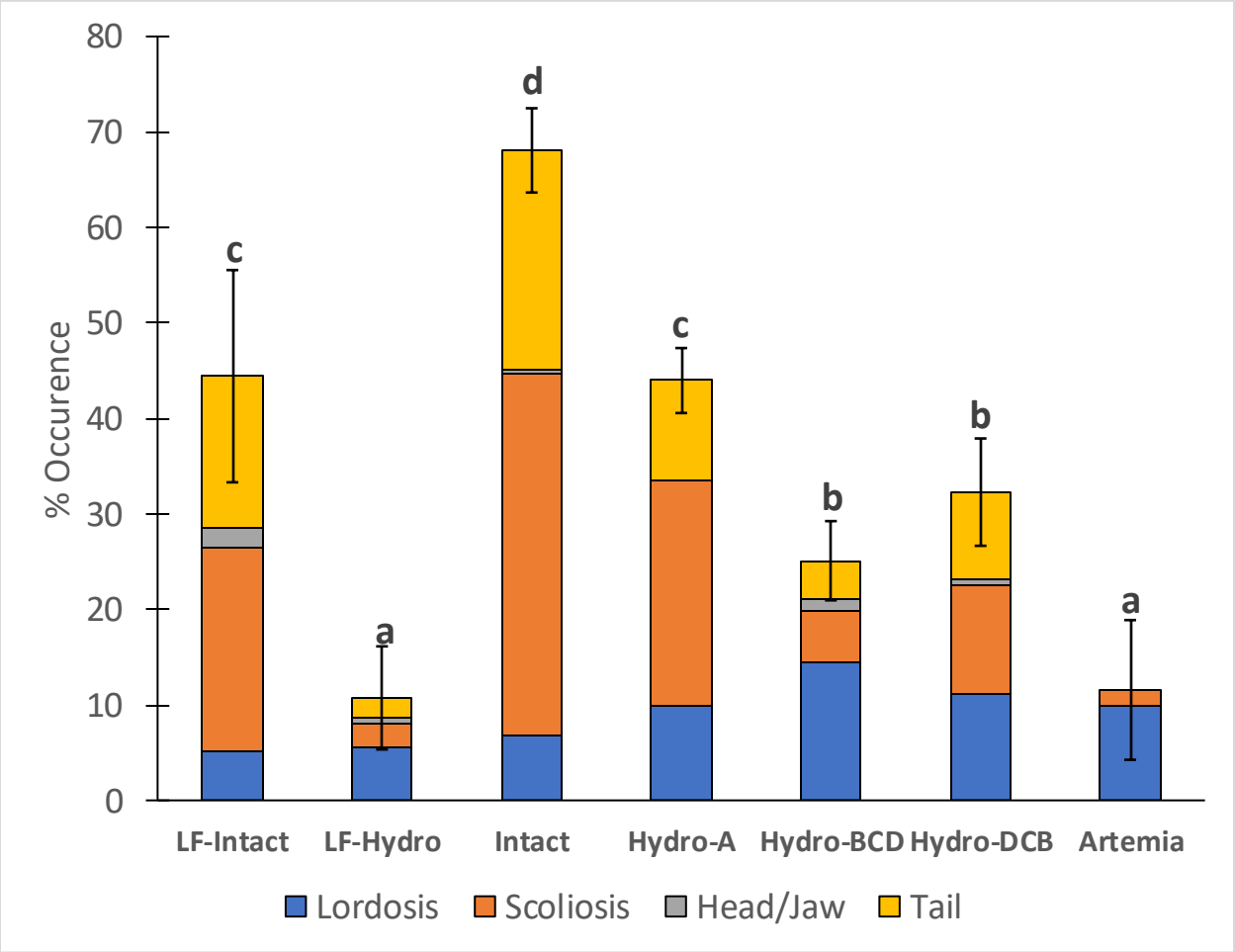

Figure 2.6. Diet effect on the occurrence of skeletal deformities (Lordosis, scoliosis, and head/jaw and tail deformities). Values are presented as means and error bars represent standard deviation for total deformities. Superscript letters indicate statistical significance between groups. The significance was determined using a One-Way ANOVA and a Tukey test with a p value < 0.05.

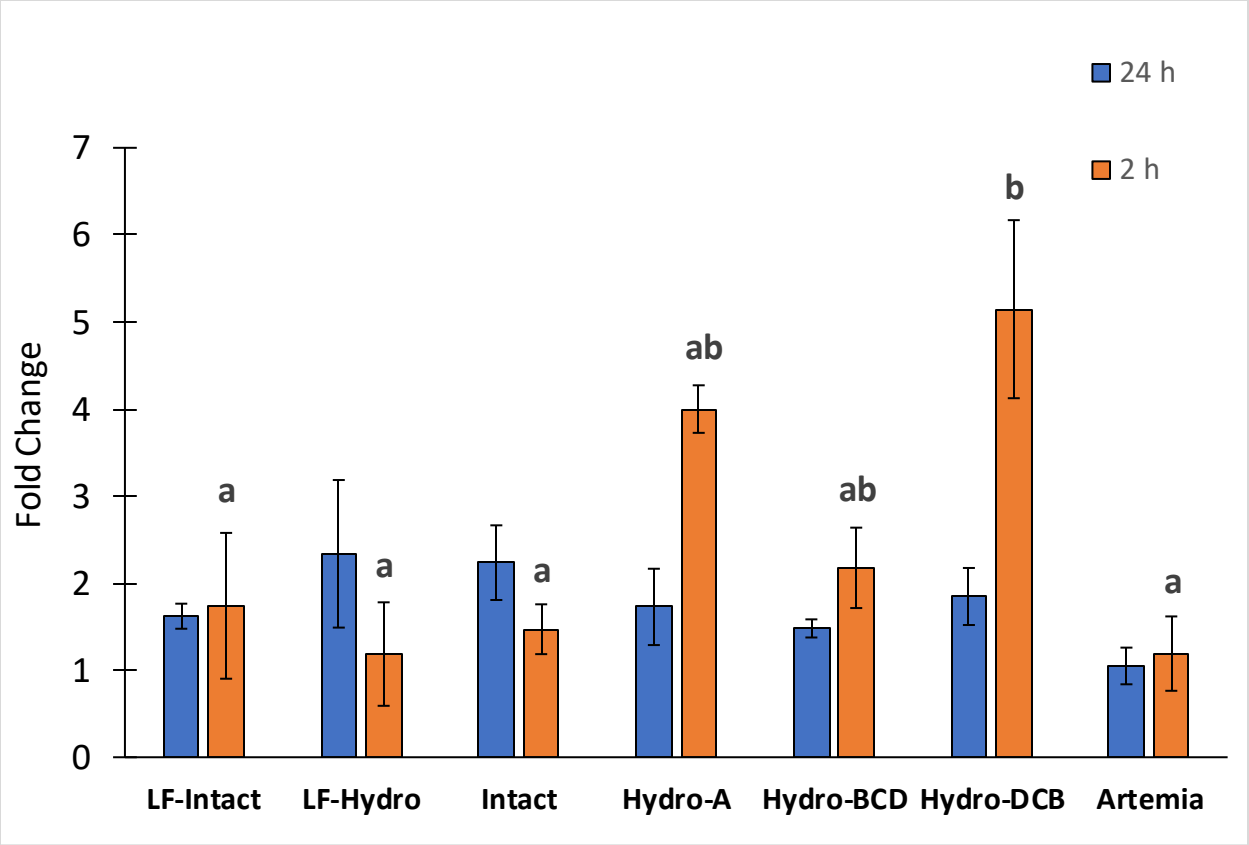

Figure 2.7. Relative expression of peptide transporter PepT1.

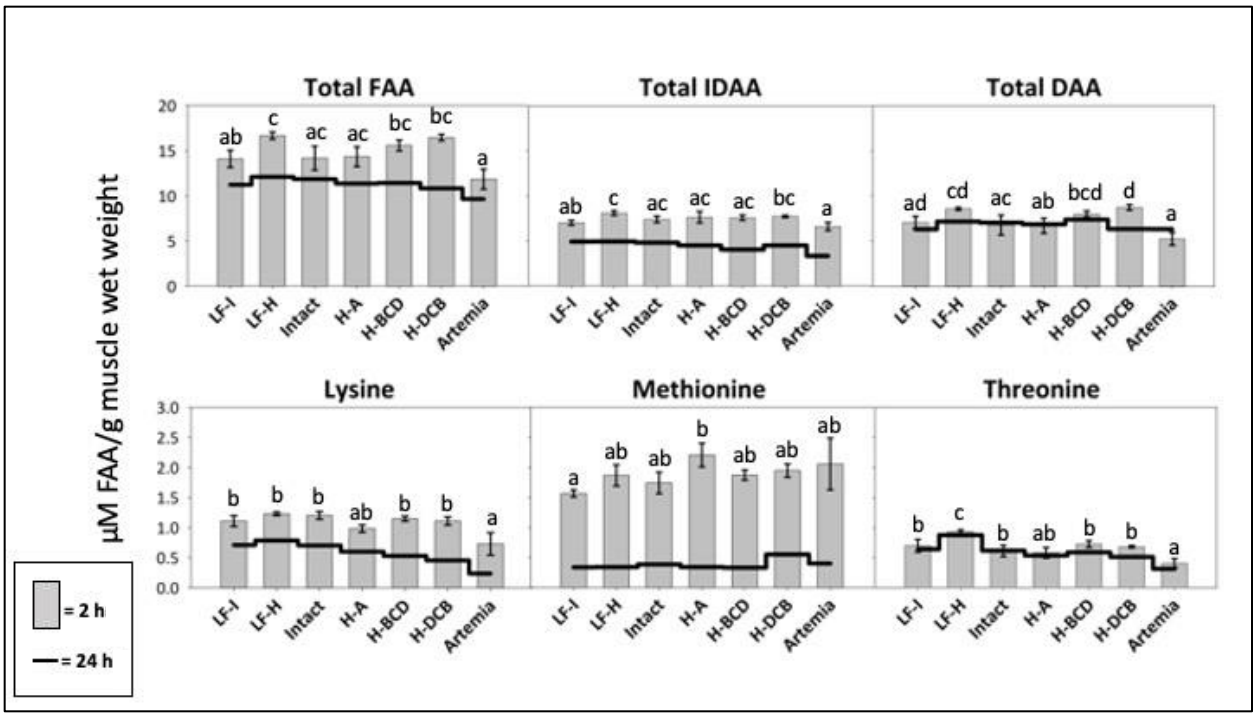

Figure 2.8. Muscle FAA levels in LMB after 3 weeks of feeding. The gray bars represent FAA levels 2 h after feeding. The black line indicates FAA physiological baseline. Different letters indicate statistical difference at $p < 0.05$.

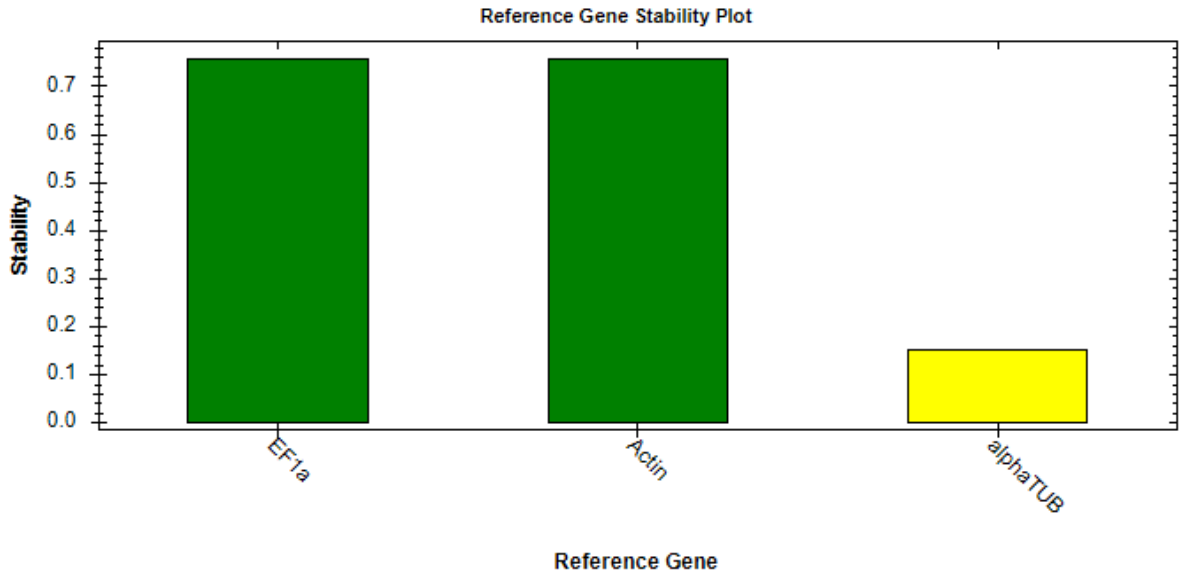

Figure 3.1. Results of stability analysis of the housekeeping genes *eEF1a1*, *α-tubulin*, and *β-actin*.

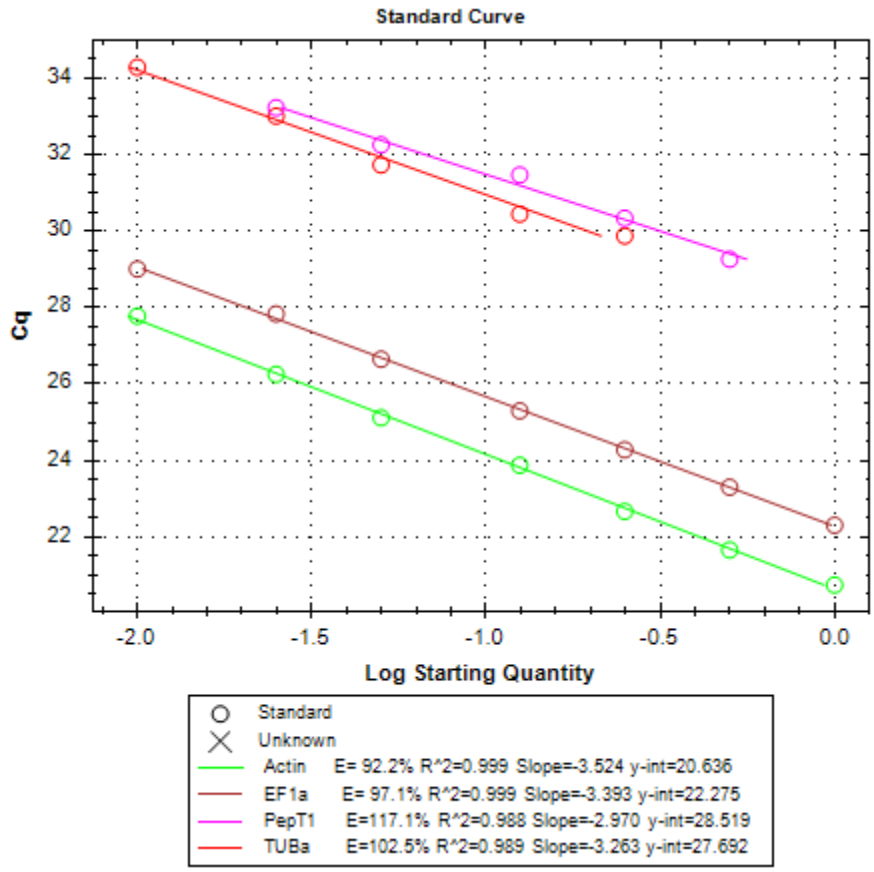

Figure 3.2. Results of amplification efficiency of each pair of primers for the target and housekeeping genes *eEF1a1*, *α-tubulin*, *β-actin*, and *PepT1*.

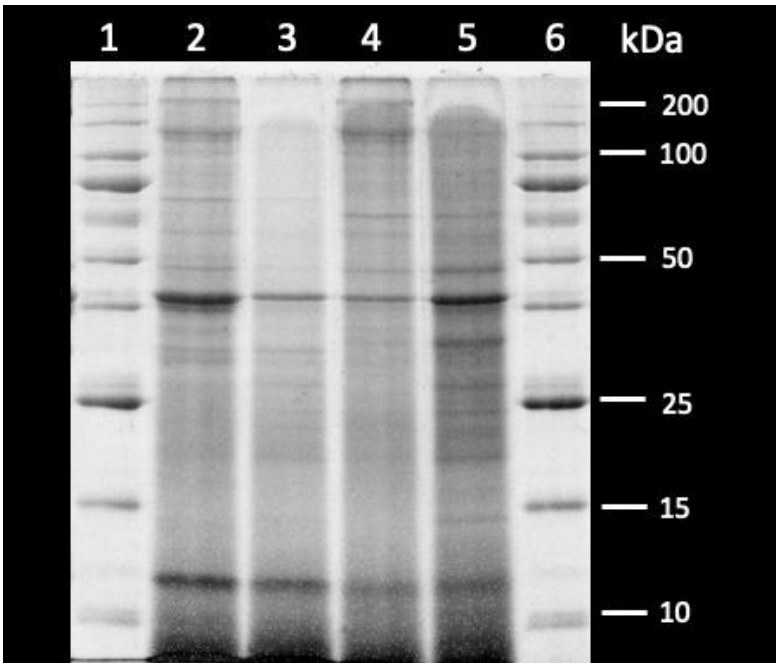

Figure 3.3. Representative reducing 12.5% T SDS-PAGE of dietary protein profile of experimental diets, represented by a 50:50 mix of the hydrolysate and intact protein produced with the corresponding muscle and enzymes. Lanes (1) broad range molecular weight standard (200–10 kDa); (2) W-W; (3) W-T; (4) T-W; (5) T-T; (6) broad range molecular weight standard (200–10 kDa). W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes.

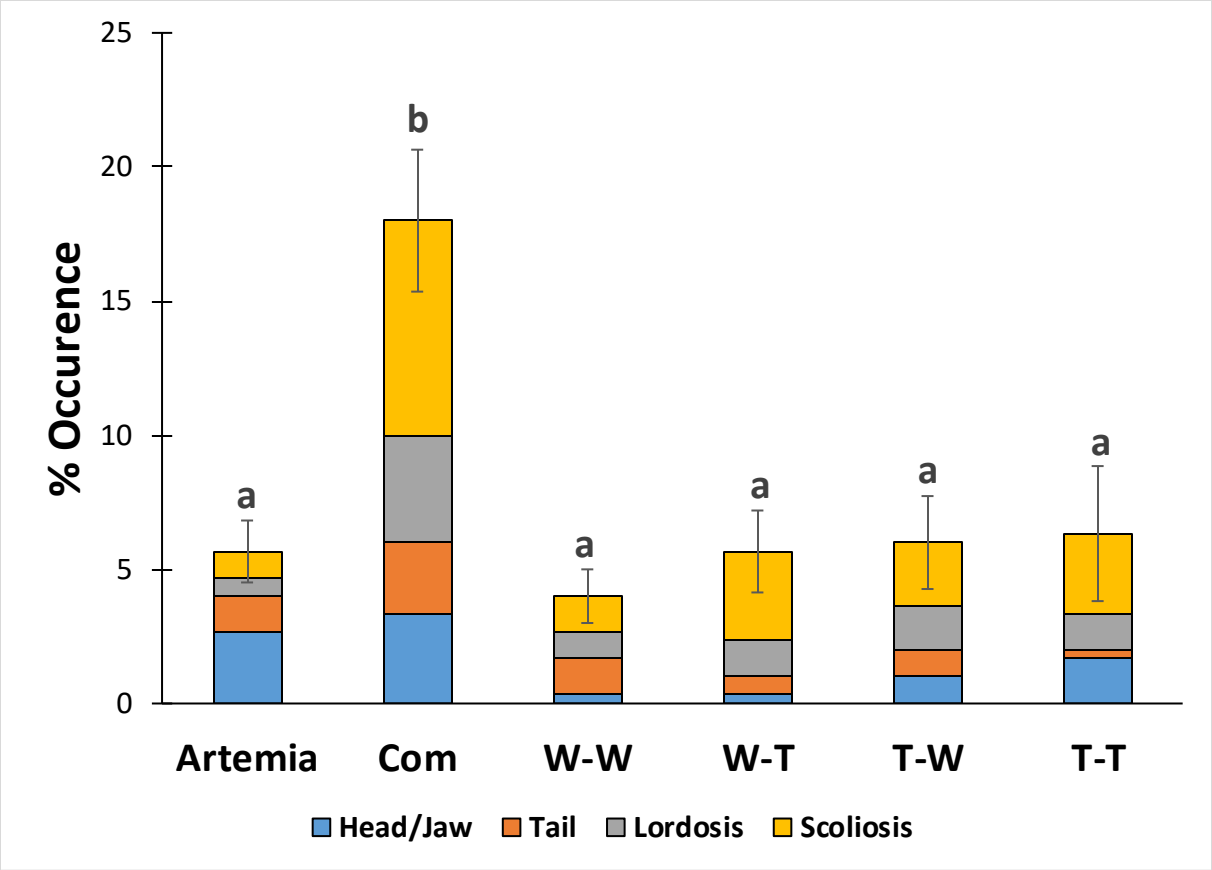

Figure 3.4. Diet effect on the occurrence of skeletal deformities (Lordosis, scoliosis, and head/jaw and tail deformities). Values are presented as means and error bars represent standard deviation for total deformities. Superscript letters indicate statistical significance between groups. The significance was determined using a One-Way ANOVA and a Tukey test with a p value < 0.05. W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes.

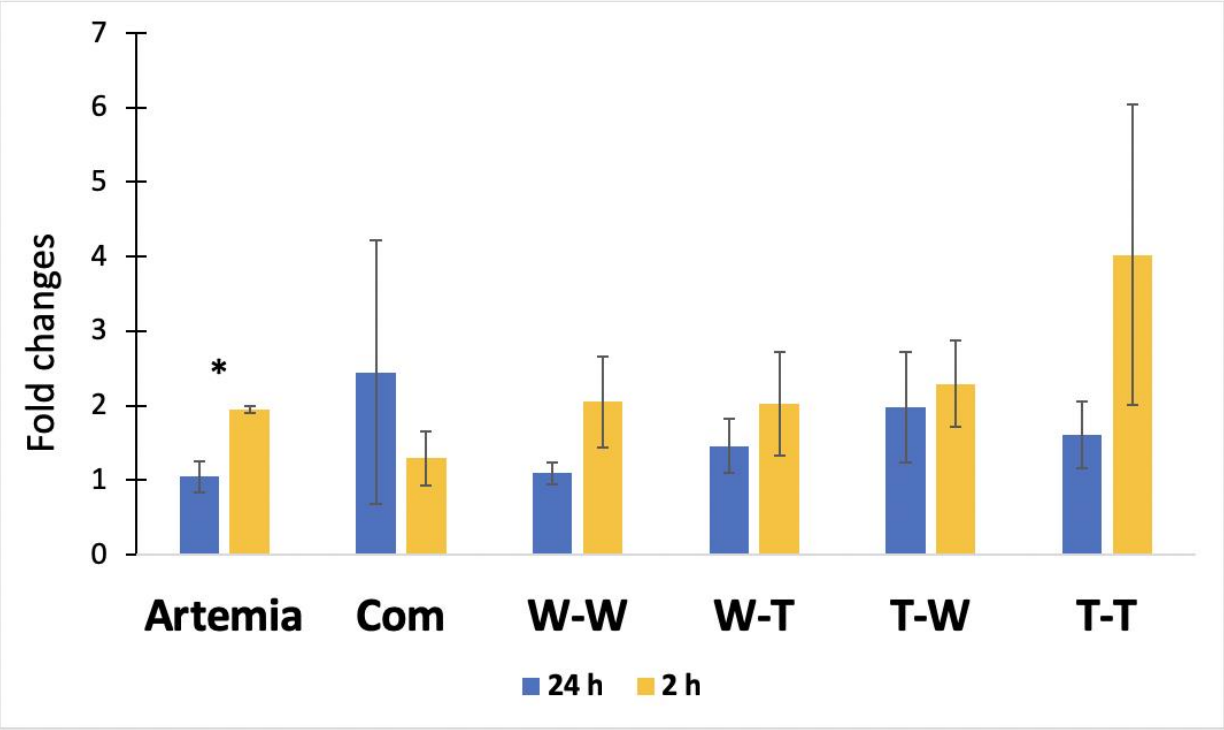

Figure 3.5. Results of qRT-PCR analysis on PepT1 expression. Values are presented as mean fold change and error bars represent standard error. Stars indicate statistical significance ($p < 0.05$) between 24 h and 2 h expression. W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes.

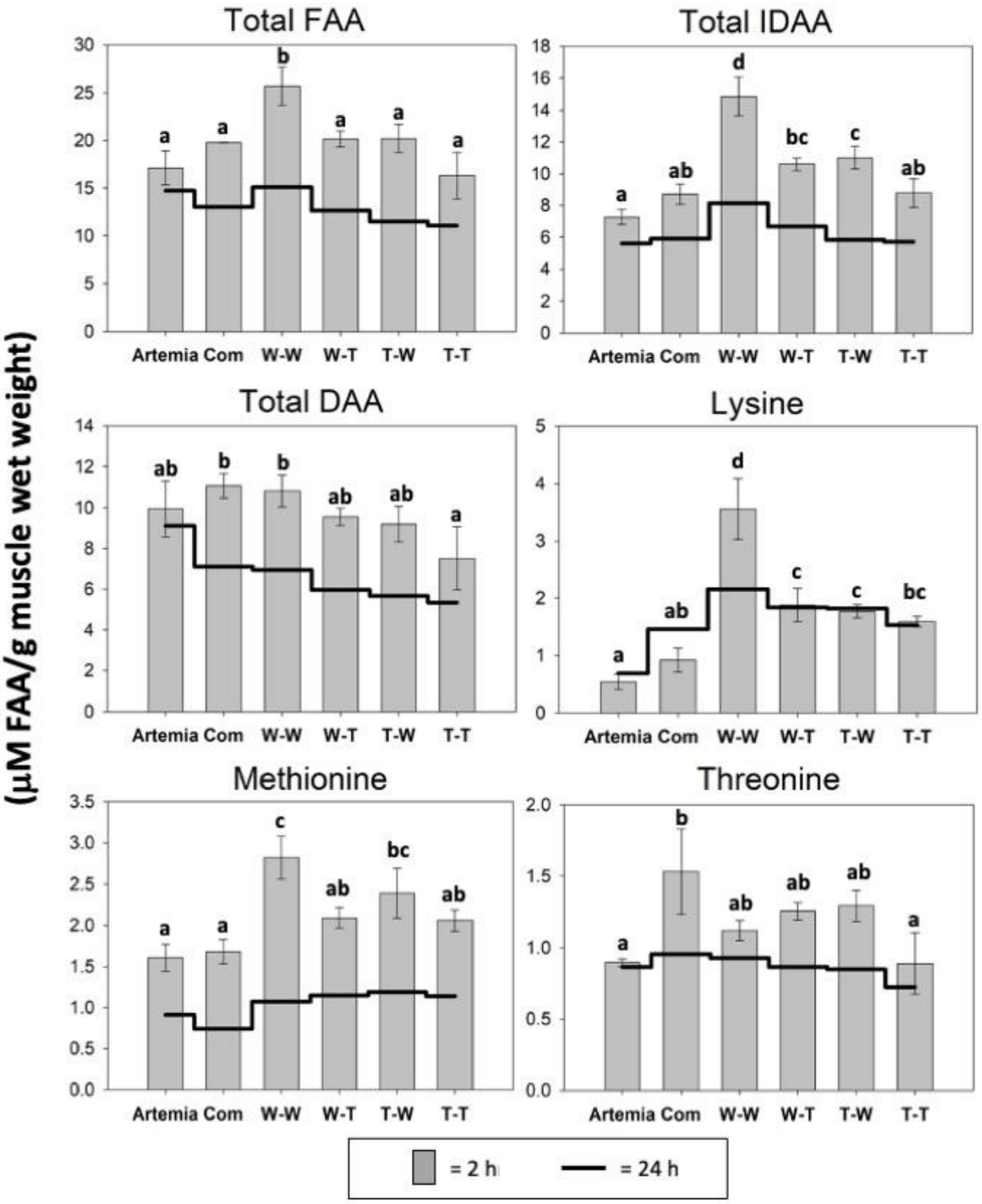

Figure 3.6. Muscle FAA levels in Walleye after 3 weeks of feeding. The gray bars represent FAA levels 2 h after feeding. The black line indicates FAA physiological baseline (24 h after feeding). Different letters indicate statistical difference at $p < 0.05$. W-W: Walleye muscle with Walleye enzymes; W-T: Walleye muscle with Tilapia enzymes; T-W: Tilapia muscle with Walleye enzymes; T-T: Tilapia muscle with Tilapia enzymes.

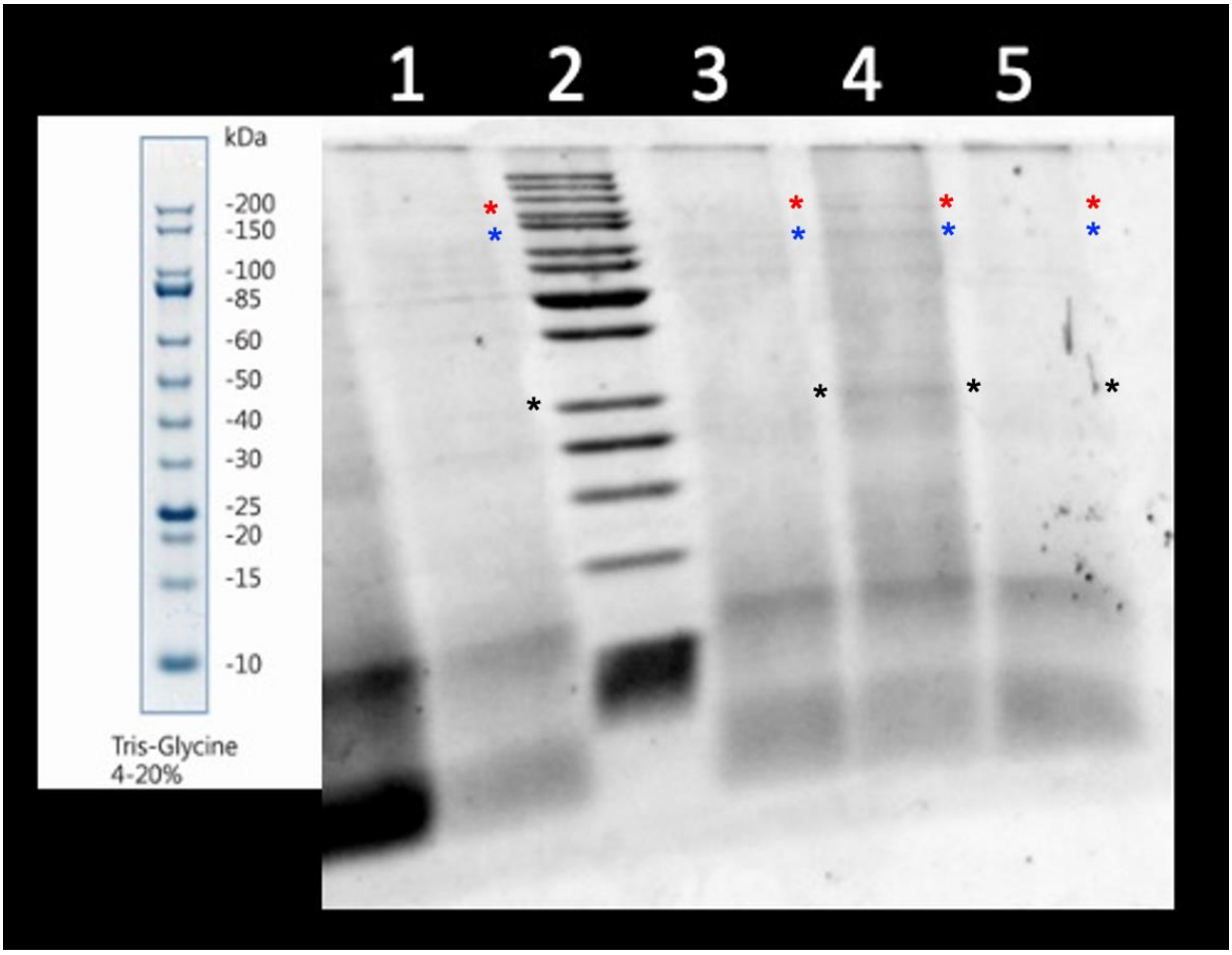

Figure 4.1. Representative reducing 12.5% T SDS-PAGE of Zebrafish protein products. Lanes (1) 2 h hydrolysate; (2) broad range molecular weight standard (200–10 kDa); (3) 1 h hydrolysate; (4) unhydrolyzed; (5) 3 h hydrolysate. Colored asterisks marked protein bands in the unhydrolyzed sample that are observed to disappear in the hydrolysate samples.

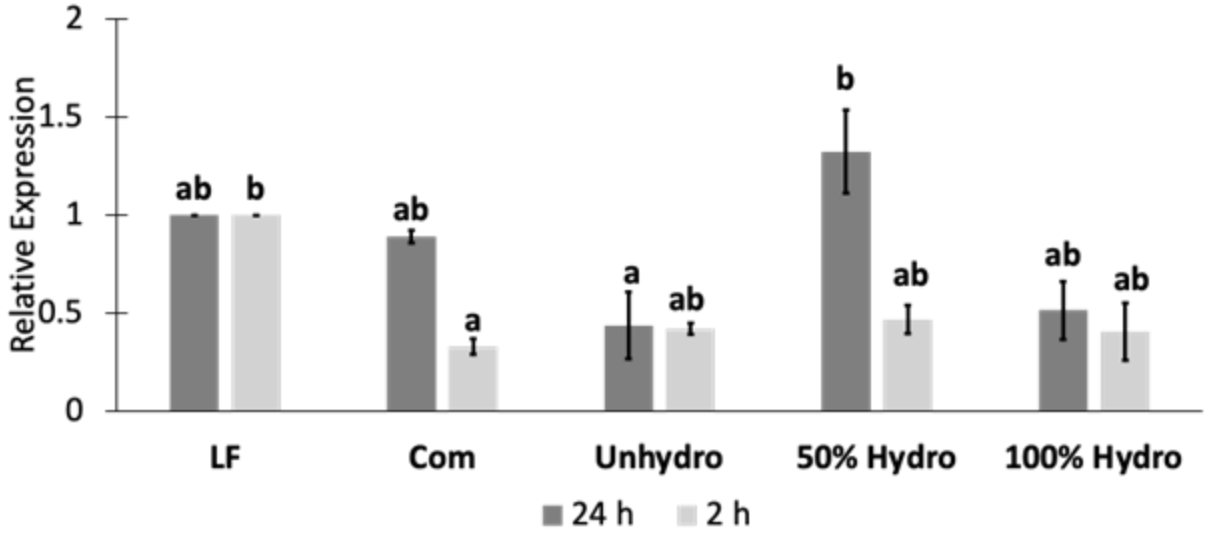

Figure 4.2. PepT1 expression. Values are presented as mean fold change and error bars represent standard error. The letters indicate statistical significance ($p < 0.05$) among 24 h and 2 h expression. The significance was determined using a One-Way ANOVA and a Tukey test at $p < 0.05$.

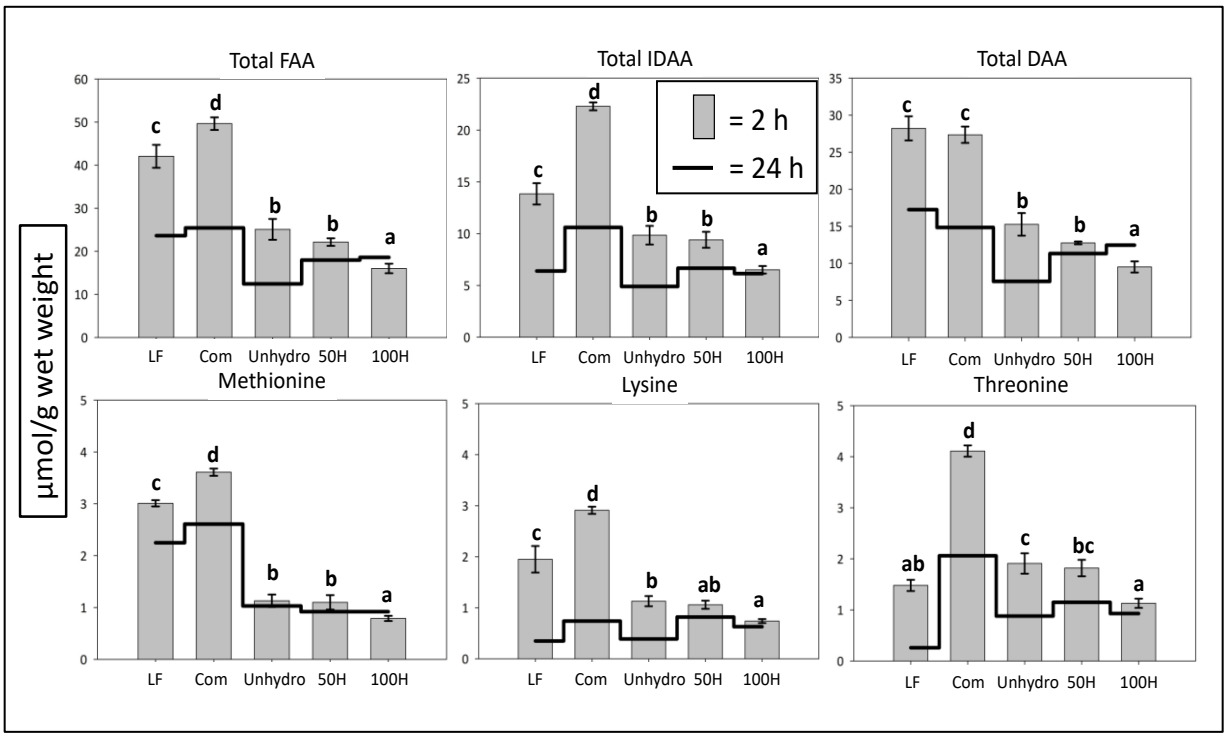

Figure 4.3. Muscle FAA levels in Zebrafish. The gray bars represent FAA levels 2 h after feeding. The black line indicates FAA physiological baseline. Values are presented as mean level and error bars represent standard deviation. Different letters indicate statistically significant difference at $p < 0.05$.

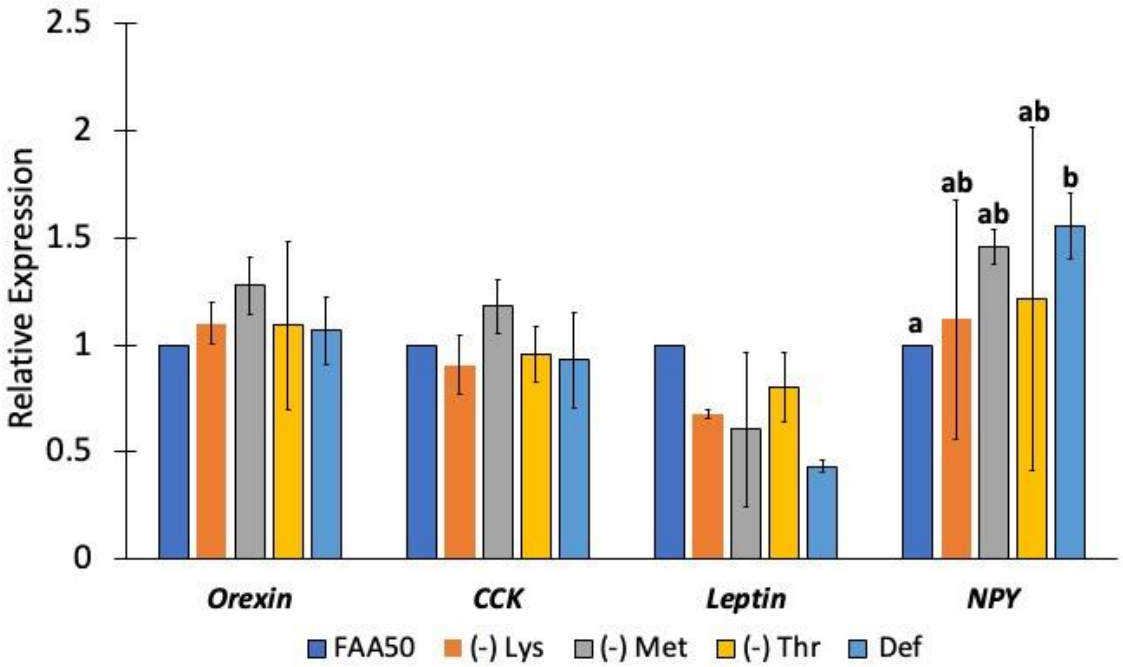

Figure 5.1. Relative hormone expression in the brain. Relative expression of the group is represented as a fold change relative to the FAA50 group (fold change = 1). Values provided are mean fold change + S.E.M (standard error of the mean). Results of One-way ANOVA and LSD test are shown on graphs when significant.

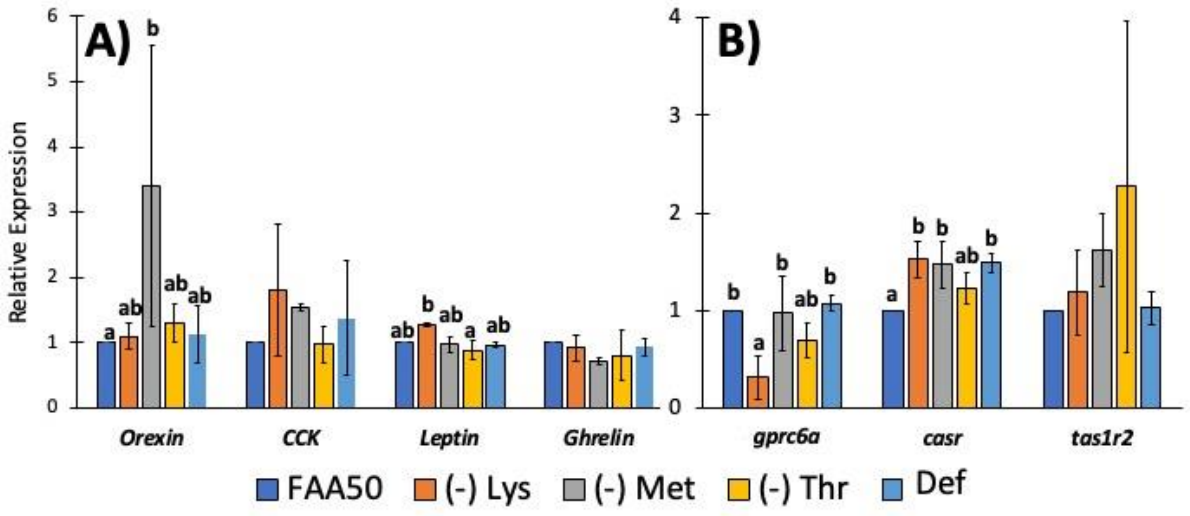

Figure 5.2. Relative hormone **(A)** and nutrient-sensing receptor **(B)** expression in the gut. Relative expression of the group is represented as a fold change relative to the FAA50 group (fold change = 1). Values provided are mean fold change + S.E.M (standard error of the mean). Results of One-way ANOVA and LSD test are shown on graphs when significant.

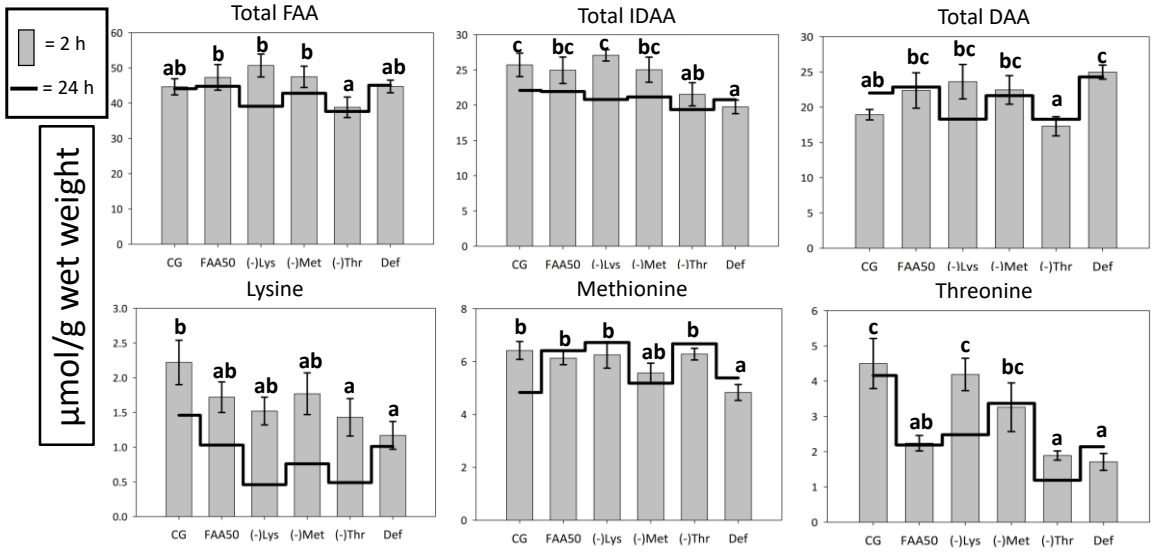

Figure 5.3. Muscle FAA levels in Zebrafish. The gray bars represent FAA levels 2 h after feeding. The black line indicates FAA physiological baseline (24 h after feeding). Different letters indicate statistically significant difference at $p < 0.05$.

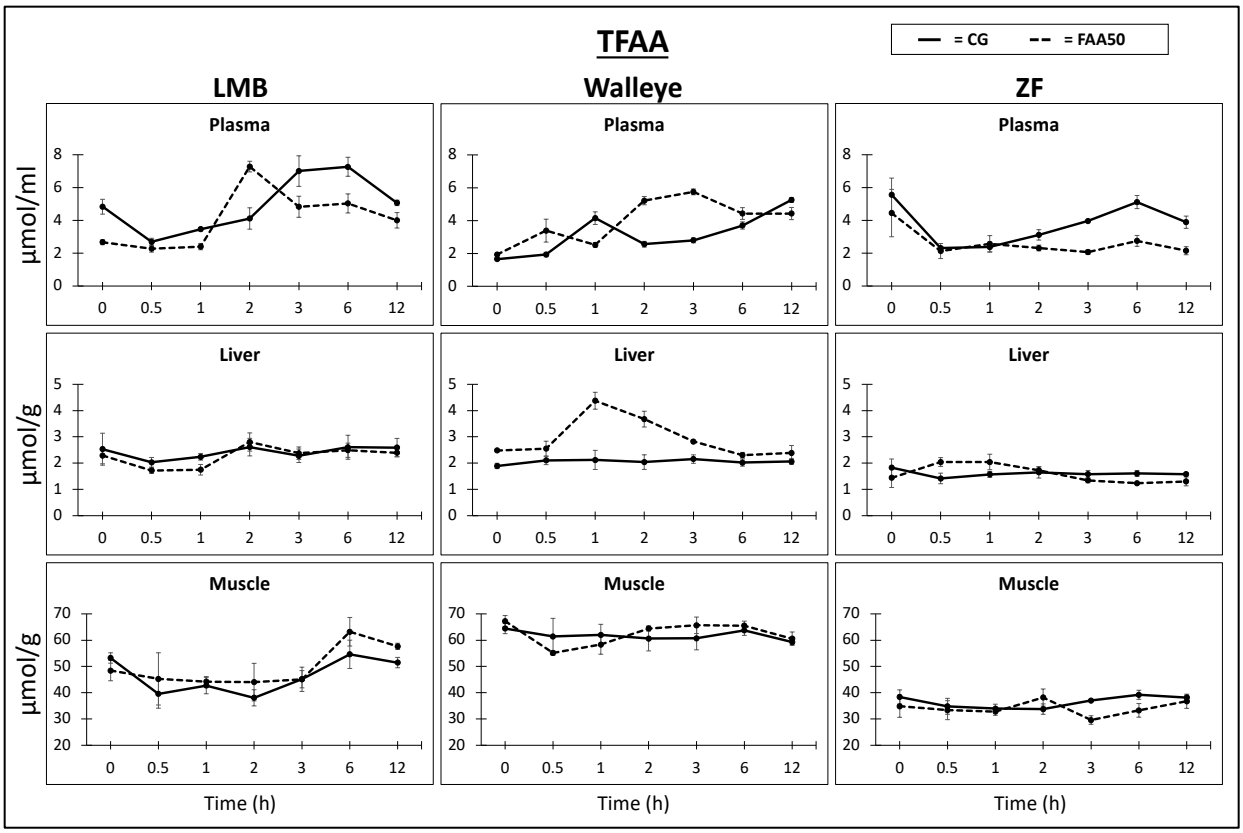

Figure 6.1. Postprandial time course of total free amino acids (TFAA) in the plasma, liver, and muscle of Largemouth Bass (LMB), Walleye, and Zebrafish (ZF). Each plot contains two postprandial patterns, representing the levels of TFAA in the specific species-FAA pool combination in response to the CG diet (solid line) and FAA50 diet (dashed line). Each plotted point represents the mean of the three samples, with error bars indicating standard deviation.

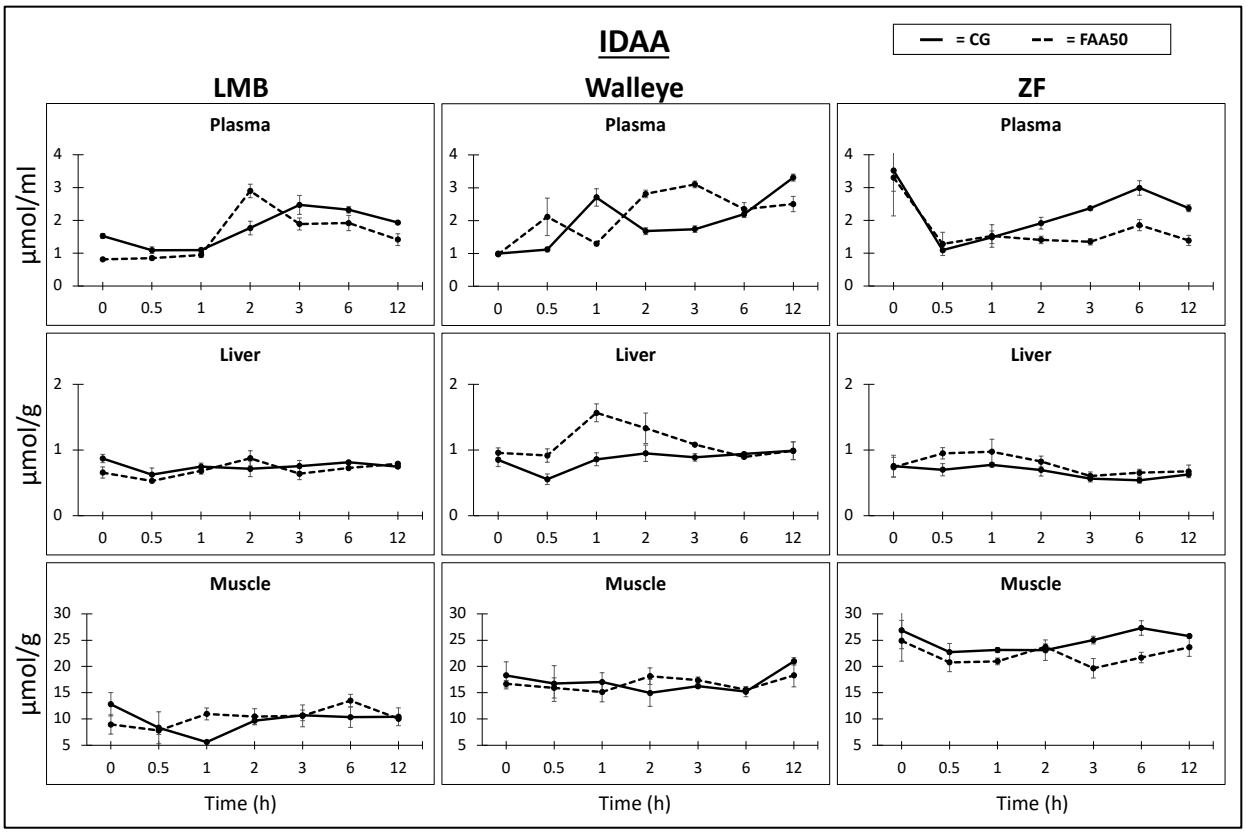

Figure 6.2. Postprandial time course of total indispensable amino acids (IDAA) in the plasma, liver, and muscle of Largemouth Bass (LMB), Walleye, and Zebrafish (ZF). Total IDAA = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Each plot contains two postprandial patterns, representing the levels of IDAA in the specific species-FAA pool combination in response to the CG diet (solid line) and FAA50 diet (dashed line). Each plotted point represents the mean of the three samples, with error bars indicating standard deviation.

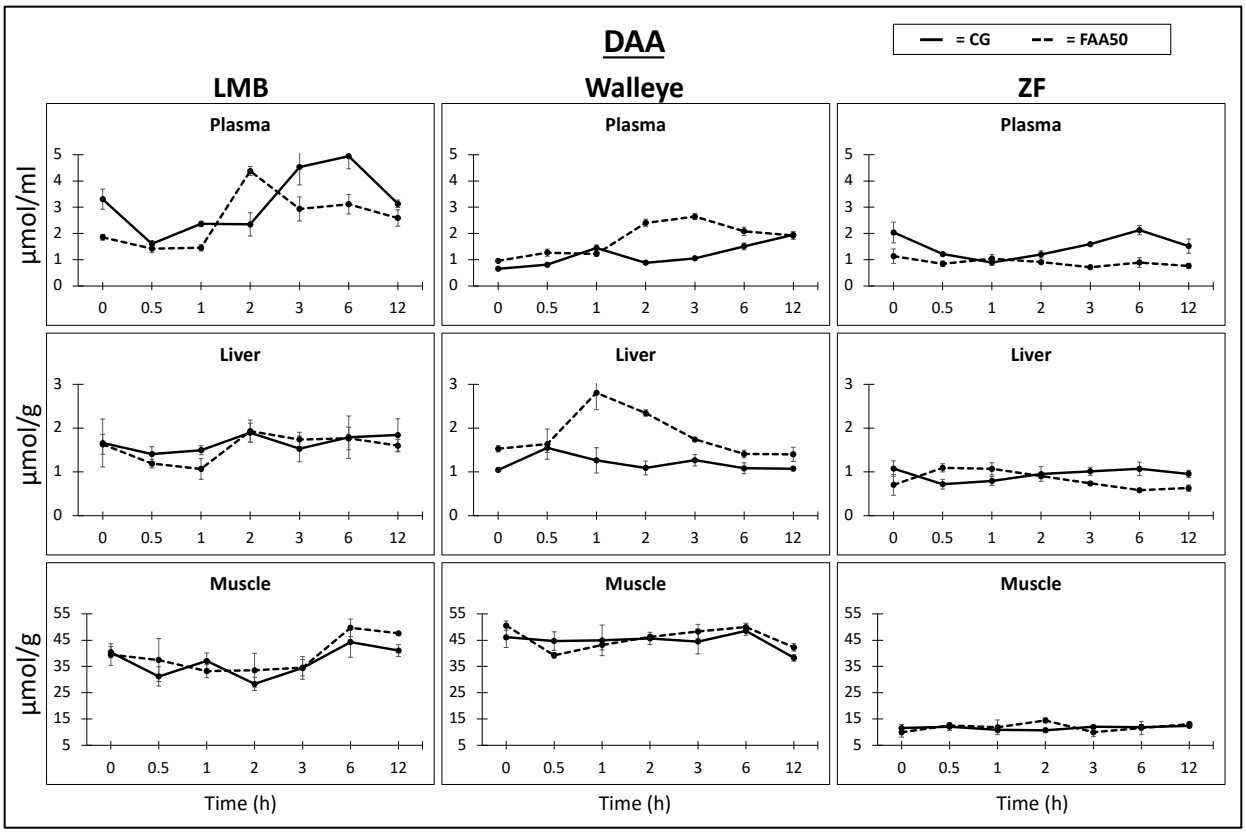

Figure 6.3. Postprandial time course of total dispensable amino acids (DAA) in the plasma, liver, and muscle of Largemouth Bass (LMB), Walleye, and Zebrafish (ZF). Total DAA = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. Each plot contains two postprandial patterns, representing the levels of DAA in the specific species-FAA pool combination in response to the CG diet (solid line) and FAA50 diet (dashed line). Each plotted point represents the mean of the three samples, with error bars indicating standard deviation.

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APPENDIX A

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