NUTRITIONAL ROLES OF GLUTAMATE AND GLUTAMINE IN THE GROWTH OF JUVENILE HYBRID STRIPED BASS

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2019

Major Subject: Animal Science

ABSTRACT

Both feeding and *in vitro* tissue studies were conducted to test the hypothesis that glutamate (Glu) and glutamine (Gln), consisting of >10% of dry weight in fishmeal (FM), may be nutritionally essential for maximal growth and survival of juvenile hybrid striped bass (HSB) (*Morone chrysops* × *Morone saxatilis*). The first *in-vitro* study, using radio-labeled tracers, determined oxidation rates of Glu, Gln, leucine, glucose, and palmitate in the liver, proximal intestine, kidney, and dorsal skeletal muscle tissue that were incubated in a Krebs-Henseleit bicarbonate buffer (pH 7.4, with 5 mM D-glucose and 1 nM insulin) containing 0, 2 mM Glu, 2 mM Gln, 2 mM leucine, 2 mM palmitate, or a mixture of those nutrients (2 mM each). $^{14}CO_2$ was collected to calculate the rates of substrate oxidation. Using the same solutions, the tissues' uptake rates of each of those nutrients were also measured by counting intracellular radioactivity. The activities of some key enzymes related to the metabolism of Glu, Gln, leucine, glucose, and palmitate were also determined in the HSB tissues. The second experiment was conducted to investigate the potential of replacement of FM in HSB diets by a mixture of poultry by-product meal (PBM) and soybean protein concentrate (SPC) that contained 37% more Glu and Gln than FM.

In the *in vitro* study, Glu and Gln were more actively oxidized in the proximal intestine, liver, and kidney of fish than the oxidation of glucose and palmitate. Together, Glu,Gln, and leucine contributed to about 80% of ATP production in fish tissues. Fish tissues had high activities of glutamate dehydrogenase (GDH), glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) as well as high rates of Glu uptake, which provided a biochemical basis for the extensive catabolism of Glu. In the FM replacement study, up to 75%

FM in HSB diet could be replaced by a mixture of PBM, SPC, and AA supplements without any adverse effect on the HSB's growth performance. Interestingly, HSB that were fed a diet with 25-50% FM replacement with poultry by-product meal and SPC exhibited better growth performance than HSB that were fed a 60% FM diet, possibly due to the bioavailability of Glu and Gln in SPC.

Collectively, results from the present study indicate that Glu and Gln are conditionally essential for the growth and health of juvenile HSB. These findings not only advance our basic understanding of AA nutrition in fish, but will also have important implications for formulating economically and environmentally sustainable aquafeeds.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Wu, and my committee members, Dr. Bazer, Dr. Burghardt, Dr. Johnson, and Dr. Mackenzie, for their guidance and support throughout the course of this research.

Thanks also go to my friends and colleagues and faculty and staff of the Department of Animal Science for making my time at Texas A&M University a great experience.

Finally, thanks to my mother and father for their encouragement and to my wife for her patience and love.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Professors Guoyao Wu (advisor) and Fuller W. Bazer of the Department of Animal Science, Professor Robert C. Burghardt of the Department of Veterinary Integrative Biosciences, Professor Gregory A. Johnson of the Department of Veterinary Integrative Biosciences, and Professor Duncan S. MacKenzie of the Department of Biology.

All work conducted for the thesis was completed by the student independently.

Funding Sources

Graduate study was supported by a research assistantship from the Department of Animal Science, Texas A&M University.

This work was also made possible in part by GUANGDONG YEUHAI FEEDS GROUP CO., LTD under Grant Number # M1701955. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the GUANGDONG YEUHAI FEEDS GROUP CO., LTD.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background

Amino acids (AAs) that are synthesized by animals had traditionally not been considered as nutritionally essential for animals, including fish. However, over the past 25 years, there has been growing interest in the physiological roles of AAs for roles other than protein synthesis in humans and other animals (Wu 2013a). The syntheses of many low-molecular-weight substances [e.g., nitric oxide (NO), polyamines, glutathione, creatine, melanin and heme] require AAs, including those that are synthesized by animal cells. In 1912, an AA was classified as nutritionally "essential" (EAA) or "nonessential" (NEAA) based on the growth and nitrogen balance of mammals. Nutritionally nonessential AAs have been previously thought to be dispensable in diets because they are synthesized *de novo* in animals. However, the amount of AAs synthesized in the body may not be sufficient to meet metabolic needs of animals, such as maximal growth in the young and optimal health in the life cycle. Additionally, nitrogen balance is not a sensitive indicator of optimal dietary requirements of adults for all AAs (Wu 2013a). In recent years, a new nutritional concept of functional AAs (FAAs) has been proposed to formulate new generations of improved diets that incorporate NEAAs (Wu 2010). Functional AAs are defined as those AAs that regulate key metabolic pathways to improve health, survival, growth, development, lactation, and reproduction of organisms (Wu 2010). A deficiency or imbalance of FAAs may impair protein synthesis, metabolism, and homeostasis in the whole body of animals. For example, supplementing glutamine, glutamate, and aspartate to a conventional diet may improve the integrity of the intestinal epithelium by providing additional

metabolic energy and substrates of synthetic processes for optimal intestinal growth and maintenance in pigs and rats (Hou et al. 2015; Wu et al. 2014). Also, an inadequate supply of arginine from the maternal diet impairs fetal development and growth in gestating swine (Mateo et al. 2008; Liu et al. 2012; Wu et al. 2018) and rats (Wu et al. 2013a).

There is growing interest in the roles of FAAs in the nutrition of both mammals and fish (Andersen et al. 2016). Dietary requirements of protein for fish range from 30% to 60% of dietary dry matter based on their species, age, size, and feeding habits (Wilson 2003). Such requirements are much greater than those for mammals and birds such as swine (12-20%), chickens (14-22%), and cattle (10-18%) (Kaushik and Seiliez 2010; NRC 2010, 2012; Wu et al. 2014). However, protein content (14-18%) or the composition of AAs in the whole body of fish is similar to that of terrestrial animals such as pigs, cattle, rats, and chickens (Lobley et al. 1980; Smits et al. 1988; Latshaw and Bishop 2001). Several reasons have been postulated to explain the high dietary protein requirements of fish. First, the basal energy needs of fish are less than those of terrestrial animals due to their poikilothermic and ammoniotelic life mode (Kaushik and Seiliez 2010). Thus, the need for dietary substances (e.g., lipids and carbohydrates) as substrates for ATP production is lower for fish, which results in a higher content of protein in fish diets than the diets of land animals. However, this explanation is not satisfactory because the oxidation of AAs, like fatty acids and glucose, can also produce ATP in fish. Second, fish may have a lower ability to utilize glucose and fatty acids, and therefore these nutrients may be easily stored in the body as glycogen and triacylglycerols, respectively, that impair the functions of tissues and cells. For this reason, a higher content of dietary protein may be necessary to prevent metabolic dysfunction in fish. Third, the contribution from AAs to meeting energy requirements may be higher (Kaushik and Seiliez 2010) and is metabolically more efficient in fish than in mammals

and birds because ammonia is directly excreted from fish into the surrounding environment without a need for ATP (Kaushik and Seiliez 2010). Therefore, dietary proteins play an important role in the growth of fish (primarily protein synthesis) and their ATP production (mainly via AA catabolism). This is a reasonable explanation for a particularly high requirement of fish for dietary AAs, but direct evidence is lacking. For example, it is unknown which fish tissues utilize AAs as major metabolic fuels and how AAs are degraded in fish tissues. As with mammals, AAs also have other functions in fish (Li et al. 2009b). For example, arginine may increase resistance to *Edwardsiela ictaluri* in channel catfish through the production of its metabolite [nitric oxide (NO)] and to induce intestinal maturation via its another metabolite, spermine, in sea bass (Buentello and Gatlin 2001; Costas et al. 2011; Andersen et al. 2016). Furthermore, glutamine may affect the secretion of pituitary hormones in rainbow trout (Andersen et al. 2016).

Among the three types of macronutrients (carbohydrates, protein, and lipids), most fish do not use carbohydrates as a major energy source (Cowey and Walton 1988). However, a high rate of AA catabolism in the whole body of fish has been observed (Wilson 2003). It has been estimated that 14-85% of the energy requirement of teleost fish is provided by AAs, depending on the fish's developmental stage (Van Waarde 1983b). Fish livers and kidneys generally have high rates of AA oxidation (Ballantyne 2001), similar to mammal and bird livers and kidneys (Wu 2013b). Furthermore, AAs are the major metabolic fuels for marine fish embryos and yolk-sac larvae (Cowey and Walton 1988). Available evidence shows that the oxidation of AAs as an entity contributes to 50-70% of total energy needs of marine fish embryos and yolk-sac larvae (Rønnestad and Fyhn 1993; Rønnestad et al. 1999).

Hybrid striped bass (HSB) are the offspring of cross-breeding striped bass and white bass and are a very popular sportfish throughout the United States, particularly in large reservoirs. HSB, also known as a wiper or whiterock bass, is a cross between the striped bass (*Morone saxatilis*) and the white bass (*Morone chrysops*). The HSB was first produced in South Carolina in the mid-1960s by fertilizing the eggs of the striped bass with the sperm of the white bass. This hybrid generally refers to the original cross, namely the palmetto bass. The reciprocal cross between the female white bass and the male striped bass produced in subsequent years is called the sunshine bass. The hybrid striped bass not only gains some superior traits inherited from its parental stocks, but also shows outbreeding characteristics for the enhancement of growth performance. For example, the HSB grows faster and has a better survival rate than the striped bass and the white bass. Moreover, the HSB has a greater ability to resist disease and to tolerate various water conditions. Thus, the HSB is now widely cultured in most of the United States as both a sportfish and a foodfish. This fish has also been introduced to several other countries and regions in Europe and Asia. The annual production of HSB ranked fourth in the aquaculture industry of United States, behind catfish, salmon, and trout (Quagrainie 2015). The juvenile HSB were used in all experiments of this dissertation.

Their natural diets consist primarily of small fish and large zooplankton. Hybrid striped bass are the fifth largest aquaculture industry in the United States based on the quantity produced (10.5 million pounds) and the fourth largest based on dollar sales ($27.8 million farm value) (Lougheed and Nelson 2001). Importantly, the annual production of HSB continues to grow (Harrell 2016). Of note, HSB have also been used for food in many countries and regions in Europe and Asia.

Publications regarding HSB aquaculture started to increase substantially in the 1990s. Successes at using fishmeal (FM)-based diets to feed HSB and determining nutrient requirements for HSB have helped the aquaculture industry to expand rapidly in the southern regions of the United States. As the FM price continues to increase due to the limited resources of fish in oceans, studies on the replacement of FM by alternative sources of proteins for different species of fish have emerged over the past 25 years. For HSB, FM could be partially replaced by poultry by-product meal (50%) and soybean meal (75%) (Gallagher 1994; Rawles et al. 2006). Some benefits of AA supplementation to HSB diets have also been confirmed. For example, dietary supplementation with arginine and/or glutamine can improve the growth performance, immune responses, and intestinal morphology of HSB (Cheng et al. 2011).

As noted previously, fish have a much higher requirement for dietary protein than mammals and poultry even though the content of protein or AAs in their bodies is similar (Wu 2013a). Based on the content of carbohydrates (~20%), lipids (~10%) and protein (~50%) in the diet of HSB, dietary protein may provide substantial amounts of energy for the growth of the fish. Dietary glutamine and glutamate, traditionally classified as NEAAs, are abundant in proteins of animal and plant origins, such as FM, poultry by-product meal, and soybean meal, which are widely used as protein sources for aquafeeds (Li et al. 2011). Glutamine is a major energy source for many types of mammalian cells, including Hela cells, enterocytes, and tumor cells. There are suggestions that glutamine may serve as a major energy substrate for leukocytes and enterocytes in fish (Li et al. 2009b; Cheng et al. 2011) as in mammals (Wu 1998), but direct evidence is lacking. Glutamine supplementation could increase the growth of HSB (Cheng et al. 2012) as reported for young pigs (Wu et al. 1996) and chickens (Bartell and Batal 2007), indicating that the regular diet may not provide sufficient glutamine to either mammals and

poultry or fish. This further extends the concept of FAAs broadly to farm animals of both agricultural and biomedical importance. At present, little is known about the cell- or tissue-specific metabolism of glutamate and glutamine and or about the utilization of dietary glutamate or glutamine in aquatic animals. In addition, as noted previously, the use of specific AAs for ATP production in fish tissues is unknown. It is possible that the synthesis and catabolism of AAs are cell- and tissue-specific in fish, as in mammals and birds (Wu 2013b). This foundational knowledge can guide the formulation of new cost-effective diets for feeding fish, as well as the development of alternatives of protein sources to FM in aquaculture.

Aquaculture Nutrition

Fish is an important food that provides humans with high-quality protein and highly bioavailable minerals, especially in developing countries (Merino et al. 2012). Aquaculture has been rapidly growing at an annual rate of 7.8% worldwide between 1990 and 2010, exceeding the growth of other food sectors including poultry, pork, dairy, and grains during the same period (Troell et al. 2014). In 2012, farmed fish production reached a record of 66 million tons globally, passing the beef production of 63 million tons for the first time. In contrast to the rapid growth of aquaculture, the capture of fish in the oceans and rivers reached a plateau of 90 million tons in the mid-1990s and stayed stable thereafter.

The current state of protein production from aquaculture and capture fisheries is directly a combined result of the expansion of the world population, economic development, climate and ecosystem changes, applications of scientific research, and many other factors (Merino et al. 2012; FAO 2018). Accordingly, the yield of aquaculture production is predicted to be close to that of captured fish by 2030 or sooner (Brander 2007). Currently, the growth of aquaculture has

heavily relied on FM and fish oil, which are mainly proceeded from wild-caught small pelagics such as anchovies, sardines, and menhaden. Aquaculture will become even more important to supply animal protein in countries and regions where livestock and poultry species have high rates of morbidity and mortality due to wide-spread infectious diseases.

Fishmeal has traditionally been considered as an ingredient with highly digestible protein for aquafeed. This feedstuff contains high levels of protein, vitamins, and minerals, as well as a balanced profile of amino acids (AAs). However, the quantity of marine fisheries has a maximum potential of around 80 million tons per year (FAO 2018). Given the rapid expansion of aquaculture and the limited natural resources of FM in association with the high requirements of dietary protein for many farmed carnivorous/piscivorous fish species, global FM and fish oil supplies cannot meet the growing demand. Thus, the inclusion levels of FM in aquafeeds will have to be reduced. Clearly, it is not sustainable to feed fish with fish.

Replacement of Fish Meal with Alternative Protein Sources

Feed cost constitutes more than half of the operating cost in intensive aquaculture, and ingredients of protein sources are the most expensive part in aquafeed. Replacing FM in aquafeeds with alternative protein resources is a promising solution to reduce the use of FM and the cost of feed for producing many fish species. Other than FM, possible protein sources to meet the dietary requirement of farmed fish for high-quality protein include plant meals, domestic animal products, single-cell proteins, and insect proteins. Because of extensive scientific research in this field, FM levels in feeds for most fish species have decreased nearly by half in the past two decades. Indeed, FM-free feeds have been successfully developed for many freshwater species such as cyprinids and tilapia (Tacon et al. 2011). However, FM-free feeds have not been

achieved for carnivorous fish, such as HSB and largemouth bass, without compromising their food intake, growth, skin color or health.

It appears that many species, especially carnivorous and marine fish, show significantly lower growth performance when fed a low-FM experimental diet, even though the experimental diets appeared to be nutritionally adequate in the provision of carbohydrates, lipids, vitamins, minerals, and EAAs (Oliva-Teles et al. 2015). Deleterious effects on nutrient utilization and fish health originally reported from those FM replacement studies have been confirmed by the results of other studies. Adverse effects include lower feed conversion rate, lower digestibility, intestinal inflammation, or enteritis (Andersen et al. 2016). For example, subacute enteritis in the distal intestine of Atlantic salmon was developed in a six-week feeding experiment using soybean meal-based feed (Baeverfjord and Krogdahl 1996). Moreover, some studies showed reductions in both feed intake by fish and the apparent digestibility of protein due to changes in the palatability and physical properties of feeds (Kaushik et al. 1995). Another emerging concern is the effect of FM substitution on nutrient composition in the body and fish flesh quality. Numerous studies have shown some adverse effects of plant protein sources on the quality of fish flesh, with its color being most negatively affected by dietary plant-source proteins (Gaylord et al. 2010; Oliva-Teles et al. 2015).

Fishmeal

Fishmeal (FM) is the coarse flour made from fresh raw fish or fish parts by cooking, pressing, drying, and milling. The quality of FM is affected by many factors, including source species, processing and storage conditions, shelf life, and adulteration with other ingredients of no or lower nutritional quality (e.g., urea and feather meal). The major source of FM is harvested

small marine fish, such as anchovies, mackerel, sardines, menhaden, and herring. As noted previously, FM is a highly digestible feed ingredient for farmed animals and an excellent source of high-quality protein and fatty acids as well as highly bioavailable minerals and vitamins. Some fatty acids in FM are essential for animal growth, such as long-chain omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid (Wu 2017). The crude protein content of a high-quality FM normally ranges from 60–72% by dry weight. The properly-balanced profile of EAAs in FM makes it a highly valued protein supplement for young growing terrestrial animals. An inclusion rate of less than 10% FM in diets is beneficial for starters and weaned pigs (Stoner et al. 1990; Kats et al. 1992). The use of FM in diets can also increase the body weight, daily weight gain, and feed intake of broilers (Blair R 2010). Because the absolute amounts of feed intake by swine and poultry are high, the quantity of FM used to feed land animals is tremendous.

Globally, the majority of FM is used to feed fish due to the increased production of farmed fish and the wide use of compounded (formulated) feed for feeding them. For example, the percentage of commercial feed used for farmed marine fish has been estimated to gradually increase from 50% to 80% between 1995 and 2020. Currently, FM is the major protein source in formulated feed for marine and carnivorous fish (Olsen and Hasan 2012). Some herbivorous and omnivorous fish such as Nile tilapia (Oreochromis niloticus), common carp (Cyprinus carpio), and crucian carp (Carassius carassius) also need a relatively high level of FM in compounded feed (Olsen and Hasan 2012). Besides the abundance of AAs such as arginine, taurine, methionine, and lysine, FM has an attractive odor to fish (possibly due to the presence of trimethylamine) that other protein sources (such as plants, meat and bone meal, poultry by-products, and insects) lack. This is a major reason why the substantial or complete replacement

of FM in diets for some farmed fish is difficult even though the provision of conventional nutrients (including EAAs) from the experimental diet appears to be adequate. In addition, FM may contain a higher content of bioactive substances such as glutathione than other sources of protein ingredients (Li et al. 2011).

Over the past three decades, numerous studies have been carried out by research institutions and the aquaculture feed industry to generate detailed knowledge on the digestive processes and nutritional requirements of many farmed fish species (National Research Council 2011). Thus, the dependency of aquaculture on FM in feed to feed many fish species, including carnivorous, marine and salmon, has been dramatically reduced since 1995 (Table 1.1). For example, the FAO reported that the feed conversion ratio (FCR, feed/gain ratio) of tilapias that were fed commercial compounded feed was 2.0 in 1995 and this value has been predicted to be reduced to 1.6 by 2020 (Tacon et al. 2011).

Plant Protein Sources

Various plant feedstuffs are commonly used as alternative protein sources for the diets of farmed fish, including meals from soybean, wheat, and peas (Oliva-Teles et al. 2015). Energy density and AA content are the two main factors to be considered when replacing FM with plant protein sources in compounded feeds. Plant meals with high energy density, such as wheat meal, usually have high carbohydrate content, but carnivorous species cannot utilize carbohydrates well. Plant meals are deficient in some EAAs such as lysine, methionine and tryptophan, and contain no taurine or creatine (Li et al. 2011). Note that only animal products provide taurine and creatine (Wu 2013a). Table 1.1 (Adapted from Tacon et al. 2011) lists the nutritional requirements of five common farmed fish species (Atlantic salmon, rainbow trout, common carp,

tilapia, and catfish) for AAs, the AA composition of protein in various feedstuffs, as well as the first, second, and third limiting AAs in these feedstuffs. As shown in Table 1.2 (Adapted from Oliva-Teles et al. 2015), the most common first limiting AA in plant-source protein is usually methionine, lysine, or tryptophan. Interestingly, all plant feedstuffs, with the possible exception of cottonseed meal, are deficient in methionine, cysteine, lysine and tryptophan.

In addition to the above-mentioned nutritional drawbacks of plant protein sources, they also contain many anti-nutritional factors, including protease inhibitors, lectins, saponins, and phytate (Oliva-Teles et al. 2015). These anti-nutritional factors reduce the digestion or absorption of nutrients and may antagonize the function of AAs and vitamins in the gastrointestinal tract. To alleviate the adverse impacts of anti-nutritional factors present in plant feedstuffs, these ingredients can be chemically, mechanically, and biologically processed through methods such as heat processing, solvent extraction, dehulling, or the use of exogenous enzymes (Jobling et al. 2001; Glencross et al. 2007; Krogdahl et al. 2010). For instance, fiber (a non-starch polysaccharide) in many plant feedstuffs can be significantly reduced to increase the relative content of protein in the feedstuffs. Phytate, which is bound via the chemical bound to phosphorus, reduces the bioavailability of minerals in soybean meal and can be treated by adding phytase to feeds to increase the release of the nutrients from the feed matrix (Gatlin et al. 2007)

Table 1.1 Estimated use of commercial feed and fishmeal in feed, and feed conversion ratios between 1995 and 2020. Adapted from Tacon et al. (2011).

Species group	% in commercial feed	Average FCR[1]	% fishmeal in feed
Marine shrimps			
1995	75	2.0	28
2005	89	1.8	24
2010	95	1.6	16
2015	97	1.5	12
2020	100	1.4	8
Marine fish			
1995	50	2.0	50
2005	70	1.9	38
2010	73	1.9	26
2015	75	1.8	18
2020	80	1.8	12
Salmon			
1995	100	1.5	45
2005	100	1.3	35
2010	100	1.3	22
2015	100	1.3	16
2020	100	1.3	12
Carps[2]			
1995	20	2.0	10
2005	45	1.8	8
2010	50	1.8	2
2015	55	1.7	1
2020	60	1.6	1
Tilapias			
1995	70	2.0	10
2005	80	1.8	8
2010	85	1.7	3
2015	90	1.6	2
2020	95	1.6	1

1 Feed conversion ratio (feed/gain ratio).
2 Excluding silver carp, bighead carp, and Indian major carps.

Table 1.2 EAA profiles of fishmeal and selected alternative protein sources with limiting amino acids. Adapted from Oliva-Teles et al. (2015)

	Amino Acids														Limiting AAs		
	Protein	Arg	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Tyr	Trp	Val	Met+ Cys	Phe+ Tyr	1°	2°	3°
Species		Requirement (% protein)																
Atlantic salmon[a]		5.0		2.2	3.1	4.2	6.7	1.9	2.5	3.1		0.8	3.3	3.1	5.0			
Trout[a]		3.9		2.1	2.9	3.9	6.3	1.8	2.4	2.9		0.8	3.2	2.9	4.7			
Carp[a]		5.3		1.6	3.1	4.4	6.9	2.2	4.1	4.7		0.9	4.4	3.1	6.3			
Tilapia[a]		4.1		3.4	3.4	6.6	5.5	2.4	3.8	3.8		1.0	5.2	3.4	5.5			
Catfish[a]		4.1		2.1	2.8	4.5	5.5	2.1	2.4	2.4		0.7	2.8	3.1	5.5			
Average[a]		4.5		2.3	3.1	4.7	6.2	2.1	3.0	3.4		0.9	3.8	3.1	5.4			
Feedstuffs		Content (% protein)																
Maize distillers wet grains and solubles[b]	44	3.4	2.0	2.4	3.5	12.0	2.6	1.9	4.6	3.2	4.1	0.5	4.4	3.9	8.7	Lys	Trp	Arg
Maize distillers dried grains and solubles[b]	29.5	4.3	2.0	2.7	3.8	11.6	3.0	2.0	4.8	3.7	3.9	0.8	5.1	4.0	8.7	Lys	Trp	---
Brewer's yeast, dehydrated[b]	48.6	4.4	0.9	2.0	4.6	6.2	6.3	1.5	3.6	4.4	2.7	1.1	4.9	2.4	6.3	Met+ Cys	His	---
Earthworm, dehydrated[b]	61	4.5	1.0	2.2	3.5	6.3	7.4	4.0	5.1	4.3	4.0		5.2	5.0	9.1	His	---	---
Maize gluten meal[b]	67.3	3.1	1.7	2.1	4.1	16.1	1.7	2.4	6.2	3.4	5.1	0.5	4.6	4.1	11.3	Lys	Trp	Arg
Wheat grain[b]	12.6	4.7	2.2	2.3	3.4	6.5	2.9	1.6	4.5	2.9	2.7	1.2	4.3	3.8	7.2	Lys	Thr	---
Faba bean[b]	29	9.0	1.2	2.6	4.1	7.1	6.3	0.8	4.0	3.5	2.7	0.8	4.6	2.0	6.7	Met+ Cys	Trp	----
Lupin, blue, seeds[b]	33.8	11.0	1.5	2.7	4.2	6.9	4.7	0.7	4.0	3.4	3.6	0.8	3.9	2.2	7.6	Met+ Cys	Lys	---
Pea seeds[b]	23.9	8.4	1.4	2.5	4.2	7.1	7.2	1.0	4.7	3.8	3.1	0.9	4.8	2.4	7.8	Met+ Cys	---	---
Linseed meal, expeller-extracted[b]	34.2	9.6	1.8	2.6	4.4	5.9	3.9	1.9	4.8	3.8	2.4	1.6	5.2	3.7	7.2	Lys	---	---
Sunflower meal, solvent-extracted, dehulled and partially dehulled[b]	37.7	8.5	1.7	2.5	4.1	6.2	3.5	2.3	4.4	3.6	2.4	1.2	4.9	4.0	6.8	Lys	---	---
Sunflower meal, solvent-extracted, dehulled and partially dehulled[b]	37.7	8.5	1.7	2.5	4.1	6.2	3.5	2.3	4.4	3.6	2.4	1.2	4.9	4.0	6.8	Lys	---	---
Canola meal[c]	39	5.9	2.5	2.6	4.0	6.8	5.6	2.0	3.9	4.2	2.9	1.2	4.9	4.5	6.8	Lys	---	---

Table 1.2 Continued

	Amino Acids															Limiting AAs		
	Protein	Arg	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Tyr	Trp	Val	Met+ Cys	Phe+ Tyr	1°	2°	3°
Feedstuffs	Content (% protein)																	
Blood meal[c]	89.6	5.5	2.1	6.2	2.8	12.7	9.2	1.3	6.5	4.4	3.2	1.5	9.2	3.4	9.7	Ile	---	---
Cookie meal[c]	12.3	4.7	1.5	1.8	4.1	7.2	3.3	1.5	4.1	3.4	4.5	1.2	4.3	3.0	8.5	Lys	---	---
Rapeseed meal, solvent-extracted, low erucic, low glucosinolates[b]	38.3	6.1	2.3	2.6	4.0	6.7	5.5	2.1	3.9	4.4	3.1	1.3	5.1	4.4	7.0	Lys	---	---
Corn grain[c]	9.3	4.1	2.2	2.5	3.7	12.2	2.7	2.3	4.9	3.3	4.6	0.8	4.7	4.4	9.6	Lys	Trp	---
Cottenseed meal[c]	40.3	11.3	1.7	2.7	3.0	5.6	4.1	1.6	5.0	3.1	2.7	1.1	4.2	3.4	7.7	Lys	Thr	Ile
Feather meal[c]	82.1	7.0	5.1	1.1	4.6	8.2	2.6	0.9	4.8	4.8	2.5	1.0	7.0	6.0	7.3	Lys	His	---
Fish meal[c]	63.4	7.6	1.1	2.4	5.1	8.3	8.3	3.2	4.4	6.5	3.7	1.1	6.0	4.2	8.1	---	---	---
Meat and bone meal[c]	52	7.1	0.9	2.3	3.7	6.8	6.1	2.1	3.6	4.7	2.8	0.8	4.3	3.1	6.3	Trp	Met+ Cys	Lys
Peanut meal[c]	43.9	12.9	1.5	2.2	3.2	5.6	3.1	1.1	4.4	3.8	3.2	0.9	3.9	2.6	7.6	Lys	Met+ Cys	---
Poultry by-product meal[c]	64.3	7.2	1.6	2.0	3.6	6.5	5.3	2.2	3.7	4.4	2.9	0.8	4.5	3.8	6.5	Lys	Thr	Trp
Soybean meal[c]	43.6	7.3	1.6	2.6	4.7	7.9	6.4	1.4	5.1	4.0	3.8	1.4	4.8	3.0	8.9	Met+ Cys	---	---
Soybean meal(Dehulled) [c]	51.8	6.0	1.3	2.2	4.1	7.1	5.5	1.2	4.7	3.9	3.3	1.2	4.3	2.6	8.0	Met+ Cys	Lys	His
Sorghum grain[c]	10.1	4.1	1.9	2.3	3.8	12.0	2.2	2.0	5.0	3.2	4.5	1.0	5.0	3.9	9.5	Lys	Arg	Thr

[a] Data from NRC, 2011;
[b] Data from Feedpedia: http://www.feedipedia.org/
[c] Data from Li et al., 2011;

The most promising alternate plant protein sources to replace FM in aquafeed are protein concentrates produced from soy, wheat, and other grains, as well as oilseeds because of their high protein content (60-80%, dry matter basis) and their low content of anti-nutritional factors (Hardy 2010). For example, salmonid species of fish could be fed a diet containing up to 75% of soy protein concentrate without developing intestinal enteritis (Kaushik et al. 1995; Stickney et al. 1996; Refstie et al. 2001). Their first limiting AAs are usually lysine, threonine, and methionine. However, owing to the high cost of their manufacturing, plant protein concentrates are currently not yet economically feasible or used as feed ingredients in the aquaculture industry. Considering the limited resource of marine fish, relatively unstable FM price, and improved processing technologies, protein concentrates may have great potential as aquafeeds on a large scale. Of all concentrate meals, soybean protein concentrate is most commonly used for laboratory research.

Soybean Protein Concentrate

Based on the definition of The Association of American Feed Control Officials (Berk 1992), "soy protein concentrate is prepared from high-quality, sound, clean, dehulled soybean seeds by removing most of the oil and water-soluble non-protein constituents and must contain not less than 70% protein on a moisture-free basis." The content of most AAs in soy protein concentrate is equal to or greater than that of menhaden FM, but soy protein concentrate has a lower content of methionine and lysine than FM (Table 1.2). Of all concentrate meals, soybean protein concentrate has partially or completely replaced FM in experimental diets for many farmed fish species without compromising their growth performance. Atlantic salmon that were fed diets with 75% of total protein being replaced by soybean protein concentrate may achieve rapid growth, compared with an FM-based diet.

Growth rate of rainbow trout was not affected when they were fed an FM-free diet containing soybean protein concentrate as the sole protein source (Kaushik et al. 1995).

Animal By-product Meal

Apart from plant proteins, terrestrial animal by-product meals are considered good substitutes of FM based on their nutritional quality and competitively low prices. Animal by-product meals are made from a variety of animal organs or tissues that are left over after the principal food components have been obtained. These processed animal protein ingredients include, but are not limited to, blood meal, intestinal mucosa, feather meal, meat and bone meal, and poultry by-product meal (Wu 2017). Compared with FM, animal by-product meals have a profile of AAs more similar to those in the animal body than plant-source proteins. Notably, the content of some AAs, such as lysine, methionine, and tryptophan, in plant-source proteins is relatively low. However, the proximate composition of animal feedstuffs is highly variable depending on their raw materials, particularly those of poultry by-products and meat and bone meals. Animal by-product meals have good palatability and no anti-nutritional factors. Inclusion of animal by-product meals as protein sources in fish feeds can range up to 20-40% without compromising fish growth in many studies (Oliva-Teles et al. 2015). For safety reasons, the use of animal by-product meals in fish diets is regulated in many nations and regions.

Poultry By-product Meal

According to the definition of AAFCO, poultry by-product meal is “the ground, rendered, clean parts of the carcass of slaughtered poultry such as necks, heads, feet, undeveloped eggs, gizzards and intestines (provided their content is removed), exclusive of feathers (except in such amounts as might occur unavoidably in good processing practices)”.

The nutrient composition of poultry by-product meal is highly variable, depending on the raw materials. However, typical high-quality poultry by-product meals can have protein content between 75-90% (dry matter basis) with the relatively low content of ash and fat. Therefore, the feedstuff's proximate composition should be carefully evaluated before use. Poultry has one of the best overall profiles of AAs among all the main animal by-products.

Soybean protein and poultry by-products contain high levels of amino acids such as glutamate and glutamine (Li et al. 2011). This allows the nutritional possibility to replace FM in feed in fish diets with a combination of plant- and animal-source feedstuffs. After dietary requirements of fish for EAAs are met, some NEAAs (e.g., glutamate and glutamine) likely play an important role in sparing the need for high FM content in diets. It should be borne in mind that most non-FM source ingredients contain one or more AAs that are relatively low compared with FM. Therefore, the insufficiency of particular AAs in the feeds with FM being replaced by alternative protein sources may be corrected by supplementing the feed with crystallized AAs.

Functional Amino Acids

There are more than 700 amino acids in nature, but the number of amino acids that exist in proteins are mainly about 20 and they are called proteinogenic amino acids. Numerous studies showed that proteinogenic AAs not only serve as building blocks for protein synthesis, but also play many other crucial roles in the metabolism and physiology of animals. The traditional classification of EAAs and NEAAs based on whether they can be synthesized *de novo* is not adequate to address the importance of their functions in animals, including fish (Hou et al. 2015). Therefore, the new concept of "functional amino acids (FAAs)" has been proposed (Wu 2010) as noted previously and is now widely accepted in the scientific community. Functional AAs are defined as "those AAs that participate in and

regulate key metabolic pathways to improve health, survival, growth, development, lactation, and reproduction of the organisms" (Wu 2010). Therefore, a functional AA can be nutritionally essential or non-essential. The main goal of using FAAs is to maximize the efficiency of animal growth and production and to optimize animal health.

Roles of functional AAs in fish nutrition and health have also been documented in many studies. These roles include the regulation of gene expression, reproduction, osmoregulation, and metamorphosis; provision of the bulk of ATP for the small intestine; activation of protein synthesis; control of appetite and body composition; modulation of immune response; and prevention of infectious disease (Andersen et al. 2016). When an FAA is supplemented to animals, the pattern of all other AAs in diets may not need to be adjusted.

Glutamate, Glutamine and Their Related Enzymes for Metabolism

Glutamate is one of the most abundant AAs in feeds (Li et al. 2011). It is also an important AA to constitute proteins because of its chemical structure. The negative charge of glutamate can stabilize the structure of protein by forming ionic bonds (Brosnan and Brosnan 2013). Glutamate was previously thought to be an NEAA because it is synthesized *de novo*. There may be several metabolic pathways to synthesize glutamate *in vivo* (Hou and Wu 2018). For example, glutamate can be formed from α-ketoglutarate and ammonia by glutamate dehydrogenase or from α-ketoacids by aminotransferases. Glutamate can also be produced from glutamine by glutaminase. Furthermore, the metabolism of most AAs (including arginine, proline and histidine) via a series of enzymes yields glutamate (Wu 2013a). Of note, in cells lacking mitochondria and thus glutaminase, glutamine cannot replace glutamate.

Many studies across different animal species have shown that a large amount of dietary glutamate is metabolized within the small intestine (foregut), primarily by

enterocytes. For example, 95-97% of dietary glutamate is metabolized by the small intestine of young pigs in the first pass (Wu 1998), and 74% of dietary glutamate was metabolized in the first pass by premature human infants on enteral feeding (Haÿs et al. 2007). Glutamate, glutamine, and aspartate are the major energy fuels for mammalian enterocytes (Wu 1998). Thus, because of the extensive first-pass catabolism of glutamate in the small intestine, the concentration of glutamate in plasma is usually low and is not affected substantially by dietary glutamate intake (Brosnan and Brosnan 2013). The gastrointestinal tract receives a glutamate signal for the presence of protein digestion by activating taste receptors in the tongue, stomach, and small intestine. Dietary glutamate can also activate umami taste receptors and further increase their appetite (Wu 2013a). Furthermore, glutamate serves as an excitatory neurotransmitter in the central nervous system to induce food intake.

Fish have a remarkably high ability to use dietary protein as an energy source (Van Waarde 1983b). For the carbon skeletons of AAs to enter the Krebs cycle and generate ATP, the amino group of AAs is ultimately liberated as ammonia through many metabolic pathways. Transaminases and dehydrogenases play critical roles in AA metabolism and ammonia production by animals (Wu 2013a). Glutamate dehydrogenase (GDH) of the liver and white muscle of goldfish showed much greater activity than other dehydrogenases and the enzymes of the purine nucleotide cycle (Van Waarde and Kesbeke 1982). Notably, the activity of GDH is particularly high in the intestine of fish. Besides GDH, the glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT), whose activities are generally high in animal tissues, also participate in AA degradation. Table 1.3 shows the activities of GDH, GOT, and GPT in the liver of some young animals.

Table 1.3 Activities of GDH, GOT, and GPT in the livers of young animals

Animal	Enzyme	Activity[a]	Reference
Pig	GDH	385	Bush et al. 2002
Red drum	GDH	200	Chan 2016
Tilapia	GDH	462	Bhaskar 1994
Tilapia	GPT	463	Abdel-Tawwab et al. 2010
Tilapia	GOT	300	Abdel-Tawwab et al. 2010
Atlantic salmon	GDH	185	Rossignol et al. 2011
Atlantic salmon	GPT	250	Fynn-Aikins et al. 1995
Atlantic salmon	GOT	230	Fynn-Aikins et al. 1995
Chicken	GDH	50	Lee et al. 1972

[a] nmol/min per mg protein

Like glutamate, glutamine is another AA that is abundantly present in all vertebrates (Wu 2013a). Glutamine is endogenously synthesized from glutamate and ammonia by glutamine synthetase. Glutamine is deaminated into glutamate plus ammonia by phosphate-activated glutaminase. Due to their chemical structures and abundances, glutamine and glutamate play an important role in ammonia detoxification (Albrecht and Norenberg 2006). However, compared with GDH, GPT, and GOT, the activities of glutaminase and glutamine synthetase in fish tissues are low except for the brain, as shown in Table 1.4 (Adatpted from Chamberlin et al. 1991). Therefore, glutamate may play a more important role in the detoxification of ammonia in the ammonotelic teleost. In chicken skeletal muscles, intracellular glutamine levels are positively related to protein synthesis (Watford and Wu 2005). High rates of protein synthesis have also been reported for rat skeletal muscle perfused with high levels of glutamine (MacLennan et al. 1987) and for chick skeletal muscles incubated with elevated levels of glutamine (Wu and Thompson 1990). There is also unequivocal evidence that dietary supplementation with glutamine enhances the growth of fish and increases protein content in their intestine (Yan and Zhou 2006; Cheng et al. 2011, 2012). In some tissues, such as the small intestine of pigs, glutamine can replace glutamate

due to the presence of glutaminase, but glutamate cannot replace glutamine because of the very low activity of glutamine synthetase (Haynes et al. 2009).

Table 1.4 Glutaminase and glutamine synthetase activities in Bowfin and Lake char fish tissues. Adapted from Chamberlin et al. (1991).

Tissue	Bowfin		Lake Char	
	Glutaminase	GS	Glutaminase	GS
Red muscle	2.2	0.86	2.4	0.09
White muscle	1.2	1.37	1.2	0.11
Brain	26.8	72.0	22.2	118
Gill	1.4	1.80	3.0	1.42
Heart	4.4	1.08	7.6	0.28
Liver	2.6	0.78	2.2	0.80
Kidney	3.0	1.45	3.2	0.80
Intestine	1.4	1.08	1.8	0.81

Values, expessed as nmol/min per mg protein, are calculated based on the content of protein (10%) in tissues.
GS, glutamine synthetase

Starch in Diets for Fish

Dietary starch is a major source of energy for human and domestic animals and may help to lower FM content in fish diets by sparing some AAs. It has been reported that fish do not have requirements for dietary starch, but some evidence suggests that an appropriate amount of dietary starch can confer a protein-sparing effect in many species of fish (Hemre et al. 2002; National Research Council 2011). However, the ability of fish to utilize dietary starch varies greatly among different species. In general, the maximum inclusion of starch in diets is 15-25% for marine or carnivorous fish but can be up to 50% for herbivorous and omnivorous species (National Research Council 2011). In animals, starch is digested in the small intestine to generate glucose, which is either oxidized to CO_2 or converted into macromolecules such as glycogen and glycoproteins (Wu 2017).

Culturing of Fish for Fish Nutrition Research

Current fish nutrition studies are mainly conducted via two systems: the indoor recirculating aquaculture system (RAS) and outdoor ponds. The RAS is a system in which water is partially reused after undergoing treatment (Ebeling and Timmons 2012). This system is used to rear fish in indoor tanks because it provides fish with a controllable and stable living environment. In order to maintain healthy fish, the RAS needs a continuous supply of clean incoming water with an optimal temperature and an optimal level of dissolved oxygen. Therefore, the RAS essentially consists of those sub-systems: tanks, a filtering system, a temperature control unit (water heater and temperature monitor), an air supply system (air pump, air tubing and air-stone), and a water recirculating system (water pump and pipes) (Figure 1). The filtering system itself contains mechanical filters, bio-filters, and UV lights to remove particles (e.g., feces and leftover feed), detoxify harmful waste products (e.g., ammonia and nitrite), and kill pathogens, respectively. Clean water is added to each tank only when the accumulated waste materials need to be removed or when the system's water volume is low due to evaporation and splashing. About one-third of the water in a tank is replaced with fresh water when the water is changed (usually every day) to maintain sufficient oxygen in the remaining water.

Compared with open ponds, using the RAS for fish nutrition studies provides various benefits. First, the conditions of the water are easily controlled and stable. Thus, the RAS minimizes the impact of irrelevant factors, and the living environment of fish will not confound their response to dietary treatments. Second, the indoor RAS prevents intruders (e.g., predators and prey of experimental fish) because the facility is a closed system. Third, the RAS allows for ease of management, harvest, and feeding because the system rears fish at a high density without compromising their health. However, the RAS does have disadvantages compared with studies conducted in open ponds. For example, it is labor-

intensive and expensive to maintain daily tanks with clean water. In addition, the RAS limits the number of large fish or sub-adult fish in a tank.

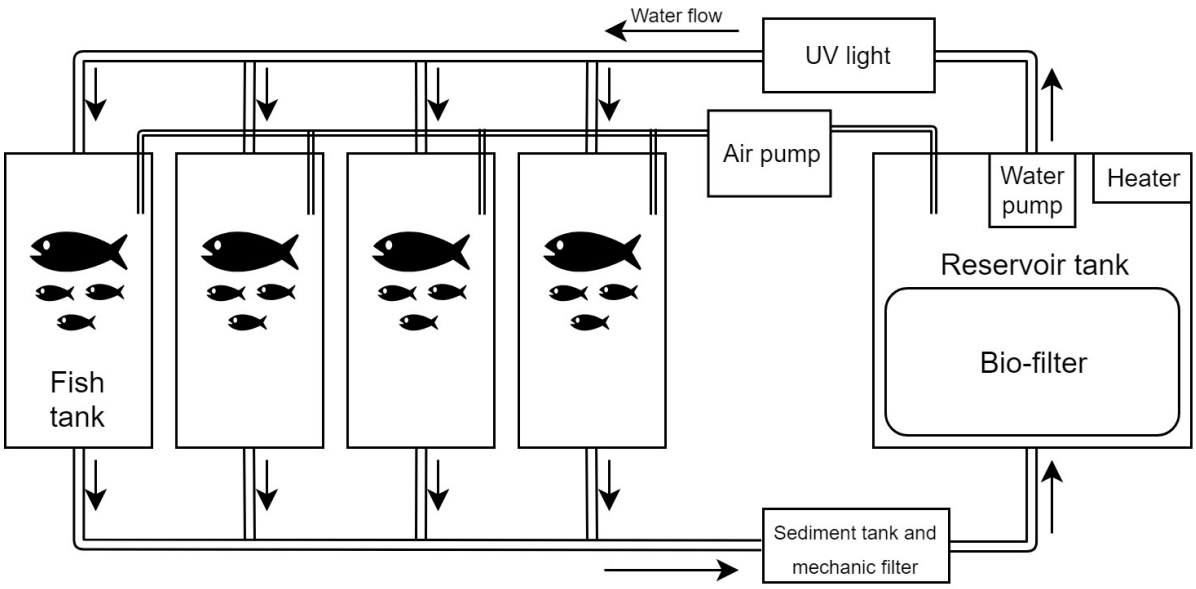

Figure 1.1 Flowchart of a recirculating aquaculture system to rear fish. The water for housing fish is prepared by mixing fresh deionized water with sea salt (1.0 – 1.5 mg/l). The salty water is added into a large reservoir tank and pumped into individual tanks through pipes. The outflowing water from each tank is collected into the sediment tank and filtered by a mechanical filter before returning to the reservoir tank. Any solids, feces, or uneaten food from the fish tanks are filtered in the sediment tank through a mechanical filter. A bio-filter is used to convert ammonia in the water into nitrite and nitrate by nitrifying bacteria. The water is then recirculated back to the fish tanks through an in-line UV light that kills pathogens, fungi, or other micro-organisms. The air pump is used to aerate water in the fish tanks and the reservoir tank via tubes connected to an air-stone. A submersible water heater in the reservoir tank is used to maintain a desired temperature of water in the fish tanks.

Novelty of This Research

Fish generally require a very high level of dietary protein (e.g. 30%-60% of dry matter, depending on species) for maintenance and growth (Van Waarde 1983a). It is thought that dietary protein provides the bulk of energy for fish. However, the ability of fish tissues to oxidize various nutrients (i.e., amino acids, glucose and fatty acids) has not been determined. . Therefore, the goals of this dissertation research are to: 1) investigate the nutritional roles of glutamine and glutamate in HSB tissues; and 2) replace FM in HSB diets with plant-source

protein that provides high amounts of glutamate and glutamine. First, the experiments of Chapter 2 revealed, for the first time, the tissue-specific oxidation of AAs, glucose, and palmitate (representatives of major energy substrates from protein, carbohydrates, and lipids, respectively) by the proximal intestine, liver, skeletal muscle, and kidneys. In Chapter 3, plant-source protein was used as an alternative to FM to provide the HSB with sufficient dietary glutamate and glutamine. The content of FM in the diets of HSB could be reduced from 60% to 15% without compromising their growth or health.

Significance

Functional AAs regulate many key biochemical pathways in animals. Whole body homeostasis as well as its physiological and metabolic regulation depend on those pathways that occur in a cell- and tissue-specific manner but are closely integrated. The results of this study are expected to: (1) define the nutritional role of glutamine and glutamate for the survival and growth of HSB; and (2) explain the need for substantial replacement of FM in diets with a mixture of poultry by-product and soy protein concentrate that provides sufficient glutamate and glutamine. Therefore, the results of this research will not only provide much-needed basic knowledge about AA metabolism in fish tissues but also guide the development of the next generation of improved and environmentally oriented aquafeeds to feed fish and other species of aquatic animals.

CHAPTER II

OXIDATION OF GLUTAMATE AND GLUTAMINE IN TISSUES OF HYBRID STRIPED BASS*

Synopsis

Fish generally have much higher requirements for dietary protein than mammals, and the reason why remains unsolved. This study was conducted with hybrid striped bass (HSB, carnivores) to test the hypothesis that they oxidize AAs at a higher rate than carbohydrates (e.g., glucose) and fatty acids (e.g., palmitate) to provide ATP for their tissues. Zebrafish were used as an omnivorous fish species for comparison. Liver, proximal intestine, kidney, and skeletal muscle tissue isolated from zebrafish and HSB were incubated at 28.5°C (zebrafish) or 26°C (HSB) for two hours in an oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4, with 5 mM D-glucose and 1 nM insulin) containing 2 mM L-[U-^{14}C]glutamine, L-[U-^{14}C]glutamate, L-[U-^{14}C]leucine, or L-[U-^{14}C]palmitate, or a trace amount of D-[U-^{14}C]glucose. In parallel experiments, tissues were incubated with a tracer and a mixture of unlabeled substrates [glutamine, glutamate, leucine and palmitate (2 mM each) plus 5 mM D-glucose]. $^{14}CO_2$ was collected to calculate the rates of substrate oxidation. The uptake of ^{14}C-labeled nutrients and the activities of key enzymes were also determined in HSB tissues to explain differences in the metabolic patterns of the nutrients among various tissues. In the presence of glucose or a mixture of substrates, the rates of oxidation of glutamate and ATP production from this AA by the proximal intestine, liver, and kidneys of zebrafish and HSB were much higher than those from glucose and palmitate. This was also true for glutamate in the skeletal muscle and glutamine in the liver of both species,

* Part of this chapter is reprinted with permission from "Amino acids are major energy substrates for tissues of hybrid striped bass and zebrafish" by Jia, S., Li, X., Zheng, S. et al., 2017. *Amino Acids*, 49: 2053-2063, Copyright 2017 by Springer Nature.

glutamine in the HSB kidney, and leucine in the zebrafish muscle in the presence of a mixture of substrates. Fish tissues had high activities of glutamate dehydrogenase, glutamate-oxaloacetate transaminase, and glutamate-pyruvate transaminase, as well as high rates of glutamate uptake, which provided a biochemical basis for their extensive catabolism of glutamate. We conclude that glutamate, glutamine, and leucine contribute to about 80% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle tissue of zebrafish and HSB. Our findings provide the first direct evidence that the major tissues of fish use AAs (mainly glutamate and glutamine) as primary energy sources instead of carbohydrates or lipids.

Introduction

Dietary requirements of protein for fish range from 30-60% based on their species, age, size, and feeding habits (Ballantyne 2001; Wilson 2003), which are much greater than those for mammals and birds such as swine (12-20%), chickens (14-22%), and cattle (10-18%) (National Research Council 2000, 2012b; Kaushik and Seiliez 2010; Wu 2014). However, protein content or the composition of amino acids (AAs) in the whole fish body is similar to that of terrestrial animals such as pigs, cattle, and chickens (Lobley et al. 1980; Smits et al. 1988; Latshaw and Bishop 2001). Several reasons have been postulated to explain the high dietary protein requirement for fish. First, the basal energy needs of fish are less than those of terrestrial animals due to their poikilothermic and ammoniotelic life mode (Kaushik and Seiliez 2010). Thus, the dietary content of lipids and starch is lower for fish, which results in a higher protein level in fish feeds. Second, the contribution from AAs towards the energy requirement may be high, and the oxidation of AAs via the Krebs cycle helps to dispose of their carbon skeletons as CO_2 and water (Weber and Haman 1996).

Therefore, dietary protein contributes to not only fish growth (protein synthesis), but also their ATP production from AA catabolism.

Among the three types of major macronutrients (carbohydrates, protein, and lipids), most fish do not use carbohydrates (e.g., starch, glycogen, and simple sugars) as a major energy source (Cowey and Walton 1988). However, high rates of AA utilization in the whole fish body have been observed (van den Thillart 1986; Jürss and Bastrop 1995; Wilson 2003; Li et al. 2009b). There is a suggestion that 14-85% of the energy requirement of teleost fish is provided by AAs, depending on the fish's developmental stage (Van Waarde 1983b). In the hepatocytes of fed and starved rainbow trout, the rates of oxidation of some AAs (alanine, serine, asparagine, and glycine) were relatively high, but the rates of oxidation of certain AAs (e.g., leucine and valine) and palmitate were low (French et al. 1981). Furthermore, AAs are the major metabolic fuels for marine fish embryos and yolk-sac larvae (Cowey and Walton 1988). Likewise, the oxidation of AAs as an entity may contribute to 50-70% of total energy needs of marine fish embryos and yolk-sac larvae (Rønnestad and Fyhn 1993; Rønnestad et al. 1999). However, to the best of our knowledge, the use of individual AAs as metabolic fuels for specific tissues of teleosts is unknown.

Zebrafish (omnivores; Laale 1977) and hybrid striped bass (HSB, carnivores; Griffin et al. 1994) are two fish species with different dietary habits. They also differ in the gastrointestinal tract because HSB have a stomach while zebrafish do not. This study was conducted with the two fish species to test the hypothesis that AAs are oxidized at a higher rate than carbohydrates (e.g., glucose) and fatty acids (e.g., palmitate) to provide ATP for their tissues.

Materials and Methods

Chemicals

The following radiolabeled chemicals were purchased from American Radiolabeled Chemicals (St. Louis, MO): D-[U-^{14}C]glucose, L-[U-^{14}C]glutamine, L-[U-^{14}C]glutamate, L-[U-^{14}C]leucine, L-[1-^{14}C]leucine, [U-^{14}C]palmitic acid, L-[N-methyl-^{14}C]carnitine, and [^{3}H]inulin. Before use, ^{14}C-Labeled glutamine and leucine were purified by using the Dowex AG1-X8 resin (acetate form, 200-400 mesh) (Self et al. 2004). ^{14}C-Labeled glutamate was purified by adding an equal volume of 1.5 M $HClO_4$ and then neutralized by a half volume of 2 M K_2CO_3. Soluene was procured from Perkin-Elmer. The liquid scintillation cocktail for determining $^{14}CO_2$ was made by dissolving 5 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis(5-phenyloxazol-2-yl) benzene into 1 L of a 1:1 mixture of toluene and 2-methoxyethanol. The sources of other chemicals, including fatty acid-free bovine serum albumin (BSA) and AAs, were as described previously (Lenis et al. 2016; Hou et al. 2016). Before use, sodium palmitate (2.5 mM) was conjugated with 0.43 mM BSA in 150 mM NaCl. Briefly, 45 ml of 5.56 mM sodium palmitate solution (in 150 mM NaCl; preheated to 70°C) was slowly added to 50 ml of 0.86 mM BSA solution (in 150 mM NaCl; preheated to 37°C). The mixed solution (containing 2.5 mM palmitate) was stirred for 1 hour at 37°C, and then adjusted to pH 7.4, and a final volume of 100 ml. After palmitate was conjugated with BSA, concentrated components (except for NaCl) of Krebs-Henseleit bicarbonate (KHB) buffer were added to the solution to obtain 2 mM palmitate, physiological concentrations of minerals [119 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$ (Wu et al. 1994)], and 20 mM Hepes (pH 7.4; Wu 1997).

Animals and Housing

Fish were housed within tanks (50 L distilled water/tank) in the Kleberg vivarium of Texas A&M University. Wild-type young adult zebrafish (*Danio rerio*) were obtained from Aquariumfish.net. Juvenile HSB (*Morone saxatilis* ♀ X *Morone chrysops* ♂) were obtained from Keo Fish Farm (Keo, Arkansas, USA). Zebrafish and HSB were maintained in two separated water cycling systems at a temperature of 28.5 and 26℃, respectively for at least a week to acclimate to local conditions. Water was circulated through mechanical and biological filters and changed regularly (30-50% daily). The system water was prepared by mixing distilled water from the Kleberg building with Instant Ocean sea salt at the salinity of 1-1.5 ppt (1-1.5 mg/L water). Air was supplied through air stones connected to air pumps, and photoperiod was maintained for 14 hours per day. The pH, ammonia, nitrite, and nitrate of the water were monitored weekly and remained within acceptable limits. Fish were fed a commercial diet (Purina) containing 40% crude protein and 12% lipids twice daily (9:00 AM and 5:00 PM). All experimental procedures were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

Collection of Tissues

On the day of tissue collection, juvenile HSB (~20 g) and adult Zebrafish (~0.5 g) were dissected 6 hours after feeding. For anesthesia, the fish were placed into water (pH 7.0) containing 40 ppt tricaine methanesulfonate (MS-222) and an appropriate amount of $NaHCO_3$. Blood samples (0.2 ml) were obtained from the tail vein of the HSB before the abdomen was opened. Thereafter, liver, proximal intestine (the front two-thirds of the whole intestine), kidney, and dorsal muscle (white muscle) samples were obtained. The proximal intestine was cut longitudinally and washed in phosphate-buffered saline to remove the remaining intestinal content, and then soft paper sheets (Kimtech) were used to dry water on

the surface of the intestine. For the measurement of carnitine palmitoyltransferase I (CPT-I) activity, fresh tissues were immediately homogenized. For the assays of other enzymes, tissues were snap-frozen in liquid nitrogen and stored at -80°C. For metabolic experiments, tissues were cut into small pieces (1 mm thickness; 4 mm in length x 2 mm in width). For determining the uptake of nutrients, tissues were sliced at 1 mm thickness.

Determination of Substrate Oxidation

Metabolic studies were conducted as described previously (Wu 1997) with some modifications. Briefly, each weighed tissue slice (15-40 mg) was incubated at 28.5℃ (zebrafish) or 26℃ (HSB) for 2 hours in 1 ml of oxygenated (95% O_2/5% CO_2) KHB buffer (pH 7.4) containing 5 mM D-glucose, 1 nM insulin, and one of the following combinations of tracer and tracee: [U-^{14}C]glucose, 2 mM glutamate + [U-^{14}C]glutamate, 2 mM glutamine + [U-^{14}C]glutamine, 2 mM leucine + [U-^{14}C]leucine, 2 mM leucine + [1-^{14}C]leucine, or 2 mM palmitate + [U-^{14}C]palmitic acid. In parallel experiments, a tissue was incubated in the presence of a tracer plus a mixture of the unlabeled substrates [i.e., 5 mM glucose (physiological concentration in fish plasma), 2 mM each of glutamate, glutamine, leucine, and palmitate)]. The concentrations of AAs and palmitate were adopted to ensure that substrates were not limiting for their oxidation in fish tissues. The specific radioactivity of each tracer in the incubation medium was approximately 2500 dpm/nmol. In all experiments, media containing the same components but no tissues were run as blanks, with 6 replicates for each radiolabeled substrate. Incubation was initiated by addition of a tissue. After a 2-hour incubation period, the reaction was terminated by addition, through the rubber stopper, of 0.2 ml 1.5 M $HClO_4$ into the incubation medium, followed by addition, through the rubber stopper, of 0.2 ml Soluene into a microtube suspended within the tube to collect $^{14}CO_2$ (Li et al. 2016). The second collection of $^{14}CO_2$ for the oxidation of [1-^{14}C]leucine was performed

by the addition of 0.7 ml of 30% (v/v) H_2O_2 into the medium to decarboxylate [1-^{14}C]α-ketoisocaproate. ^{14}C radioactivity was measured in the liquid scintillation cocktail using a Packard scintillation counter (Self et al. 2004). Based on the rates of $^{14}CO_2$ production from a labeled substrate, the tissues used in our study were viable during a 2-hour incubation period (data not shown). The medium and tissue in each tube was stored at -20°C for later determination of concentration of metabolites of alanine, aspartate, glutamate, ammonia, and lactate.

Determination of Metabolites in the Homogenates of Tissue and Incubation Medium

Alanine, aspartate, and glutamate were determined using HPLC methods involving precolumn derivatization with ophthaldialdehyde after the process of homogenation as previously described (Li et al. 2010; Dai et al. 2014b). Ammonia and L-lactate was determined using spectrophotometric methods as described in Appendix A.

Determination of Uptake Rates of Substrates

The uptake of nutrients by HSB tissues was measured with the use of ^{14}C-labeled substrates as described for the oxidation experiments, except that ^{3}H-inulin was included as an extracellular marker (Li et al. 2009a; Lei et al. 2012). Briefly, a weighed tissue slice was incubated at 26 °C for 5 minutes in 1 ml medium. After the incubation period, the tissue was quickly transferred to a new petri dish with cold phosphate-buffered saline for washing. The washing step was quickly repeated 3 times to ensure that no radio-labeled substrate remained on the surface of tissue slices. Then the tissue was transferred into a 1.5-ml microtube containing 0.5 ml of 1 M NaOH for solubilization overnight. An aliquot (0.3 ml) of the solubilized tissue solution was mixed with the scintillation cocktail (Hionic-Fluor,

PerkinElmer), and ^{14}C and ^{3}H radioactivities were measured using the dual counting program in a Packard Scintillation Counter (Self et al. 2004).

Determination of Enzymatic Activities

The activities of glutamate dehydrogenase (GDH), glutamate-pyruvate transaminase (GPT), glutamate-oxaloacetate transaminase (GOT), kidney-type glutaminase (K-GLS), liver-type glutaminase (L-GLS), glutamine synthetase (GS), branched-chain amino acid transaminase (BCAT), pyruvate kinase (PK), hexokinase (HK), phosphofructokinase-1 (PFK-1), and carnitine palmitoyltransferase 1 (CPT-1) in the liver, proximate intestine, kidney, and muscle tissue were determined as described previously (Wu et al. 1991, 2000, 2011; Self et al. 2004; Li et al. 2009a). Briefly, approximately 100 mg of frozen liver, proximal intestine, and muscle tissue, along with 30-50 mg of kidney tissue, were used for homogenization. For the assays of all enzymes except BCAT and CPT-I, a tissue was homogenized with a Dounce glass tissue grinder containing 1.5 mL of a freshly prepared buffer [300 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 3 mM dithiothreitol, 0.5% (v/v) Triton X-100, and a mixture of protease inhibitors (aprotinin, chymostatin, pepstatin A, and phenylmethylsulfonyl fluoride; 5 mg/L each)] on ice. For BCAT assays, a tissue was homogenized with a buffer consisting of 50 mM Hepes (pH 7.5), 3 mM EDTA, 5 mM dithiothreitol, 2% (v:v) Triton X-100, and a mixture of protease inhibitors (aprotinin, chymostatin, pepstatin A, and phenylmethylsulfonyl fluoride; 5 mg/L each). The whole homogenates were centrifuged at 600 × g for 10 minutes at 4°C. All the supernatant fluid (containing the mitochondria and cytosol) was subjected to three cycles of freezing in liquid nitrogen and thawing in a 26°C water bath before use for enzyme assays. The protein concentration of the supernatant fluid was determined by the BCA protein assay kit (Pierce). The activities of enzymes were measured as described in Appendix A and are expressed on the basis of protein content.

The CPT-I assay was performed with the use of ^{14}C-carnitine, as described by Brown (2003). Briefly, a tissue was homogenized in 0.25 M sucrose with the Dounce homogenizer. The whole homogenates were centrifuged at 600 x g for 15 minutes at 4°C. The supernatant fluid was collected and subjected to further centrifugation for 15 minutes at 7700 x g. The supernatant fluid was discarded and the pellet (mitochondria) was resuspended in the buffer containing 10 mM potassium phosphate buffer (pH 7.0) and 1 mM dithiothreitol. The intact mitochondria were used immediately for CPT-I assay, as described in Appendix .

Calculation and Statistical Analysis

The rate of oxidation of each substrate in a tissue (CO_2/mg tissue per hour) was calculated as the radioactivity (dpm) of the $^{14}CO_2$ produced by the tissue divided by the specific radioactivity of the substrate in the incubation medium. Rates of ATP production were calculated from the rates of CO_2 production by multiplying the coefficient (ATP/CO_2) according to the following equations:

Glutamate: $C_5H_9NO_4 + 4.5O_2 \rightarrow NH_3 + 5CO_2 + 3H_2O$; 22.5 mol ATP/mol Glu or 4.5 mol ATP/CO_2

Glutamine: $C_5H_{10}N_2O_3 + 4.5O_2 \rightarrow 2NH_3 + 5CO_2 + 2H_2O$; 22.5 mol ATP/mol Gln or 4.5 mol ATP/CO_2

Leucine: $C_6H_{13}NO_2 + 7.5O_2 \rightarrow NH_3 + 6CO_2 + 5H_2O$; 34.5 mol ATP/mol Leu or 5.75 mol ATP/CO_2

Palmitate (PA): $C_{16}H_{32}O_2 + 23O_2 \rightarrow 16CO_2 + 16H_2O$; 106 mol ATP/mol PA or 6.625 mol ATP/CO_2

Glucose (Glc): $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$; 30 mol ATP/mol Glc or 5 mol ATP/CO_2

The rates of ATP production from the oxidation of substrates into CO_2 and H_2O, expressed as mol ATP/mol substrate, were glutamate, 22.5; glutamine, 22.5; leucine, 34.5;

palmitate, 106; and glucose, 30. It is assumed that ammonia is not converted into urea in HSB and zebrafish tissues. The coefficients of ATP production per mole of CO_2 produced from the oxidation of substrates, expressed as mol ATP/mol CO_2, were glutamate, 4.5; glutamine, 4.5; leucine, 5.75; palmitate, 6.625; and glucose, 5. Data were analyzed by one-way analysis of variance and the Student-Newman-Keuls multiple comparison test (Assaad et al. 2014). Log transformation of variables was performed when the variances of data were not homogenous among treatment groups, as assessed by the Levene's test. Differences between values obtained in the presence or absence of a mixture of energy substrates were determined by the paired t-test. Probability values < 0.05 were taken to indicate statistical significance.

Results

Oxidation of Amino Acids, Glucose and Palmitate in Fish Tissues

Data on the rates of CO_2 production from the oxidation of different nutrients in HSB and zebrafish tissues are summarized in Table 2.1 and Table 2.2, respectively. In the proximal intestine, liver, kidney, and skeletal muscle of HSB and in all zebrafish tissues studied except for the proximal intestine, the rate of CO_2 production from [U-^{14}C]glutamate oxidation was lower ($P < 0.05$) in the presence of a mixture of energy substrates, compared with the presence of 5 mM unlabeled glucose alone. In the proximal intestine, kidney, and skeletal muscle of both fish species, glutamate was most oxidative among the tested nutrients under all the experimental conditions. The rate of CO_2 production from [U-^{14}C]glutamine oxidation was the highest in the liver, and the second highest (after glutamate) in the kidney of HSB and zebrafish in the presence of 5 mM glucose or a mixture of substrates or in the proximal intestine in the presence of a mixture of substrates. The rate of CO_2 production from [U-^{14}C]leucine oxidation was the second highest in the skeletal muscle of both fish species,

but was the lowest in the proximal intestine and liver of HSB and in the proximal intestine and kidney of zebrafish when the tissues were incubated with a mixture of substrates. The rate of hepatic CO_2 production from [U-^{14}C]leucine oxidation differed ($P < 0.05$) markedly between HSB and zebrafish.

Table 2.1 Oxidation of a labeled nutrient by hybrid striped bass tissues in the presence of 5 mM unlabeled glucose or a mixture of unlabeled substrates

Medium mixture	Labeled nutrient				
	[U-^{14}C] Glutamate (2 mM)	[U-^{14}C] Glutamine (2 mM)	[U-^{14}C] Leucine (2 mM)	[U-^{14}C] Palmitate (2 mM)	[U-^{14}C] Glucose (5 mM)
			Proximal intestine		
Glucose[1]	21.8 ± 1.17[a]	4.46 ± 0.36[b]	0.25 ± 0.02[c]	1.61 ± 0.06[c]	3.96 ± 0.27[b]
Mixture[2]	6.69 ± 0.3[a]*	1.90 ± 0.08[b]*	0.24 ± 0.03[d]	0.84 ± 0.09[c]*	1.08 ± 0.07[c]*
			Liver		
Glucose[1]	2.25 ± 0.06[b]	3.14 ± 0.34[a]	0.38 ± 0.03[d]	0.33 ± 0.04[d]	1.29 ± 0.10[c]
Mixture[2]	1.89 ± 0.09[b]*	3.02 ± 0.34[a]	0.10 ± 0.01[d]*	0.23 ± 0.02[c]*	0.26 ± 0.02[c]*
			Kidney		
Glucose[1]	28.0 ± 3.83[a]	31.9 ± 0.54[a]	8.42 ± 0.39[b]	6.54 ± 0.49[c]	8.71 ± 0.41[b]
Mixture[2]	21.1 ± 0.45[a*]	9.47 ± 0.77[b]*	4.51 ± 0.25[c]*	3.04 ± 0.2[d]*	5.71 ± 0.32[c]*
			Skeletal muscle		
Glucose[1]	0.60 ± 0.07[a]	0.16 ± 0.03[b]	0.18 ± 0.03[b]	0.03 ± 0.01[c]	0.50 ± 0.04[a]
Mixture[2]	0.32 ± 0.04[a]*	0.04 ± 0.01[c]*	0.10 ± 0.02[b]*	0.00*	0.11 ± 0.01[b]*

Data, expressed as nmol CO_2/mg tissue per hour, are mean ± SEM, n = 6.
[1] Oxidation of a labeled nutrient in the presence of 5 mM unlabeled glucose.
[2] Oxidation of a labeled nutrient in the presence of a mixture of unlabeled substrates (2 mM glutamate, 2 mM glutamine, 2 mM leucine, 2 mM palmitate, and 5 mM glucose).
a-d: Within a row, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA.
* $P < 0.05$ vs the value in the presence of 5 mM unlabeled glucose alone.

Table 2.2 Oxidation of a labeled nutrient by zebrafish tissues in the presence of 5 mM unlabeled glucose or a mixture of unlabeled substrates

Medium mixture	Labeled nutrient				
	[U-^{14}C] Glutamate (2 mM)	[U-^{14}C] Glutamine (2 mM)	[U-^{14}C] Leucine (2 mM)	[U-^{14}C] Palmitate (2 mM)	[U-^{14}C] Glucose (5 mM)
			Proximal intestine		
Glucose[1]	15.5 ± 0.55^{a}	5.60 ± 0.64^{c}	0.58 ± 0.06^{e}	1.52 ± 0.07^{d}	7.57 ± 0.49^{b}
Mixture[2]	14.9 ± 0.79^{a}	4.24 ± 0.22^{b}*	0.22 ± 0.03^{d}*	0.68 ± 0.06^{c}*	4.31 ± 0.39^{b}*
			Liver		
Glucose[1]	6.57 ± 0.29^{b}	7.48 ± 0.59^{a}	4.24 ± 0.13^{c}	0.43 ± 0.01^{e}	2.83 ± 0.17^{d}
Mixture[2]	3.29 ± 0.26^{b}*	4.39 ± 0.31^{a}*	1.90 ± 0.29^{c}*	0.40 ± 0.06^{d}	1.65 ± 0.18^{c}*
			Kidney		
Glucose[1]	57.9 ± 1.80^{a}	17.4 ± 1.27^{b}	5.76 ± 0.30^{d}	5.61 ± 0.53^{d}	12.8 ± 0.80^{c}
Mixture[2]	23.1 ± 1.38^{a}*	5.93 ± 0.25^{b}*	2.04 ± 0.33^{e}*	3.54 ± 0.34^{d}*	4.66 ± 0.41^{c}*
			Skeletal muscle		
Glucose[1]	0.74 ± 0.03^{a}	0.55 ± 0.06^{b}	0.32 ± 0.06^{c}	0.21 ± 0.03^{d}	0.10 ± 0.01^{e}
Mixture[2]	0.31 ± 0.05^{a}*	0.10 ± 0.01^{c}*	0.20 ± 0.03^{b}*	0.06 ± 0.01^{d}*	0.06 ± 0.01^{d}*

Data, expressed as nmol CO_2/mg tissue per hour, are mean ± SEM, n = 6.
[1] Oxidation of a labeled nutrient in the presence of 5 mM unlabeled glucose.
[2] Oxidation of a labeled nutrient in the presence of a mixture of unlabeled substrates (2 mM glutamate, 2 mM glutamine, 2 mM leucine, 2 mM palmitate, and 5 mM glucose).
a-d: Within a row, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA.
* $P < 0.05$ vs the value for the presence of 5 mM unlabeled glucose alone.

The rate of CO_2 production from [U-^{14}C]palmitate oxidation was the lowest among the tested nutrients in the skeletal muscle of HSB and in the liver and skeletal muscle of zebrafish in the presence of 5 mM glucose or a mixture of substrates, and could not be detected in HSB skeletal muscle incubated in the presence of a mixture of energy substrates. In the liver of both fish species, palmitate oxidation was limited under the experimental conditions. The rates of glucose oxidation differed ($P < 0.05$) between the two fish species.

Specifically, in the presence of a mixture of energy substrates, the rate of CO_2 production from [U-^{14}C]glucose oxidation was much lower ($P < 0.05$) than that from glutamate or glutamine oxidation in the proximal intestine, liver, and kidney, and was similar to that from leucine oxidation in the HSB skeletal muscle incubated in the presence of a mixture of substrates. Under the same experimental conditions, the rates of CO_2 production from [U-^{14}C]glucose oxidation in the proximal intestine and kidney were much higher ($P < 0.05$) in zebrafish than those in HSB, making glucose the second and third most oxidative substrate in the intestine and kidney, respectively.

ATP Production from the Oxidation of Nutrients in Fish Tissues

Data on ATP production from the oxidation of nutrients by HSB and zebrafish tissues are summarized in Tables 2.3 and 2.4, respectively. Results of the comparison of ATP production from nutrients among tissues were generally similar to those for the rates of nutrient oxidation noted previously. In the presence of a mixture of energy substrates, the percentage of ATP produced from the oxidation of AAs (glutamate plus glutamine plus leucine) was 78.5%, 89.1%, 77.1%, and 80.4%, respectively, for the proximal intestine, liver, kidney, and skeletal muscle of HSB, and was 77.1%, 80.7%, 75.3%, and 77.6%, respectively, for the proximal intestine, liver, kidney, and skeletal muscle of zebrafish.

Comparisons of ATP production from nutrients among different tissues are summarized in Tables 2.5 and 2.6 for HSB and zebrafish, respectively. Kidneys from both fish species had the highest rate of ATP production per g of tissue from glutamate, glutamine, glucose, palmitate, and leucine in the presence of 5 mM glucose or a mixture of energy substrates. The proximal intestine of HSB had the second highest rate of ATP production per g of tissue for all nutrients. Based on tissue weights of 20-g juvenile HSB and 0.5-g zebrafish, the rates of ATP production from nutrient oxidation per tissue in the presence of a

mixture of substrates are summarized in Table 2.7. Glutamate produced the most ATP in the intestine, kidneys, and skeletal muscle of HSB and zebrafish, whereas glutamine was the most predominant metabolic fuel in the liver of both fish species.

Table 2.3 Production of ATP from the oxidation of a nutrient by hybrid striped bass tissues in the presence of 5 mM unlabeled glucose or a mixture of unlabeled substrates

Medium mixture	Nutrient					
	Glutamate (2 mM)	Glutamine (2 mM)	Leucine (2 mM)	Palmitate (2 mM)	Glucose (5 mM)	% of ATP from AAs
			Proximal intestine			
Glucose[1]	98.0 ± 5.25[a]	20.5 ± 1.58[b]	1.43 ± 0.12[d]	10.7 ± 0.38[c]	19.8 ± 1.36[b]	
Mixture[2]	30.1 ± 1.36[a]*	8.56 ± 0.35[b]*	1.40 ± 0.16[d]	5.55 ± 0.57[c]*	5.42 ± 0.33[c]*	78.5
			Liver			
Glucose[1]	10.1 ± 0.26[b]	14.1 ± 1.53[a]	2.16 ± 0.19[d]	2.22 ± 0.27[d]	6.47 ± 0.52[c]	
Mixture[2]	8.51 ± 0.40[b]*	13.6 ± 1.55[a]	0.56 ± 0.05[c]*	1.49 ± 0.13[c*]	1.28 ± 0.08[c]*	89.1
			Kidney			
Glucose[1]	126 ± 17[a]	143 ± 2.42[a]	48.4 ± 2.26[b]	43.3 ± 3.27[b]	43.5 ± 2.05[b]	
Mixture[2]	95.0 ± 2.05[a*]	42.6 ± 3.46[b]*	25.9 ± 1.43[c]*	20.1 ± 1.29[d*]	28.5 ± 1.61[c]*	77.1
			Skeletal muscle			
Glucose[1]	2.71 ± 0.30[a]	0.73 ± 0.16[b]	1.02 ± 0.19[b]	0.20 ± 0.05[c]	2.48 ± 0.21[a]	
Mixture[2]	1.50 ± 0.18[a]*	0.16 ± 0.02[c]*	0.56 ± 0.12[b]*	0.00*	0.54 ± 0.02[b]*	80.4

Data, expressed as nmol ATP/mg tissue per hour, are mean ± SEM, n = 6. % of ATP from AAs: percentage of ATP from the oxidation of amino acids.
[1] Oxidation of the indicated nutrient in the presence of 5 mM glucose alone.
[2] Oxidation of the indicated nutrient in the presence of a mixture of energy substrates (2 mM glutamate, 2 mM glutamine, 2 mM leucine, 2 mM palmitate, and 5 mM glucose).
a-d: Within a row, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA.
* $P < 0.05$ vs the value for the presence of 5 mM unlabeled glucose alone.

Table 2.4 Production of ATP from the oxidation of a nutrient by zebrafish tissues in the presence of 5 mM unlabeled glucose or a mixture of unlabeled substrates

Medium mixture	Nutrient					
	Glutamate (2 mM)	Glutamine (2 mM)	Leucine (2 mM)	Palmitate (2 mM)	Glucose (5 mM)	% of ATP from AAs
			Proximal intestine			
Glucose[1]	69.7 ± 2.46[a]	25.2 ± 2.86[c]	3.34 ± 0.34[e]	10.1 ± 0.45[d]	37.9 ± 2.43[b]	
Mixture[2]	67.2 ± 3.54[a]	19.1 ± 0.99[b]*	1.24 ± 0.18[d]*	4.48 ± 0.36[c]*	21.5 ± 1.93[b]*	77.1
			Liver			
Glucose[1]	29.6 ± 1.32[a]	33.6 ± 2.66[a]	24.4 ± 0.77[b]	2.87 ± 0.08[d]	14.1 ± 0.85[c]	
Mixture[2]	14.8 ± 1.16[b]*	19.7 ± 1.39[a]*	10.9 ± 1.69[c]*	2.65 ± 0.37[e]	8.23 ± 0.87[d]*	80.7
			Kidney			
Glucose[1]	260 ± 8.08[a]	78.3 ± 5.72[b]	33.1 ± 1.72[d]	37.1 ± 3.49[d]	63.9 ± 4.01[c]	
Mixture[2]	104 ± 6.19[a]*	26.7 ± 1.12[b]*	11.7 ± 1.88[c]*	23.4 ± 2.27[b]*	23.3 ± 2.07[b]*	75.3
			Skeletal muscle			
Glucose[1]	3.32 ± 0.15[a]	2.48 ± 0.28[b]	1.83 ± 0.33[c]	1.40 ± 0.18[c]	0.48 ± 0.05[d]	
Mixture[2]	1.41 ± 0.21[a]*	0.47 ± 0.02[b]*	1.15 ± 0.17[a]*	0.38 ± 0.06[bc]*	0.30 ± 0.06[c]*	77.6

Data, expressed as nmol ATP/mg tissue per hour, are mean ± SEM, n = 6. % of ATP from AAs: percentage of ATP from the oxidation of amino acids.
[1] Oxidation of the indicated nutrient in the presence of 5 mM glucose alone.
[2] Oxidation of the indicated nutrient in the presence of a mixture of energy substrates (2 mM glutamate, 2 mM glutamine, 2 mM leucine, 2 mM palmitate, and 5 mM glucose).
a-d: Within a row, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA.
* $P < 0.05$ vs the value for the oxidation of substrate alone.

Table 2.5 Comparison of ATP production from a nutrient among hybrid striped bass tissues in the presence of 5 mM glucose or a mixture of energy substrates

Tissue	Glutamate	Glutamine	Leucine	Palmitate	Glucose
In the presence of 5 mM glucose					
Proximal intestine	98.0 ± 5.25^{b}	20.6 ± 1.66^{b}	1.43 ± 0.12^{c}	10.7 ± 0.38^{b}	19.8 ± 1.36^{b}
Liver	10.1 ± 0.26^{c}	14.1 ± 1.53^{c}	2.16 ± 0.19^{b}	2.22 ± 0.27^{c}	6.47 ± 0.52^{c}
Kidney	126 ± 17^{a}	143 ± 2.42^{a}	48.4 ± 2.26^{a}	43.3 ± 3.27^{a}	43.5 ± 2.05^{a}
Skeletal muscle	2.71 ± 0.30^{d}	0.73 ± 0.16^{d}	1.02 ± 0.19^{d}	0.20 ± 0.05^{d}	2.48 ± 0.21^{d}
In the presence of a mixture of energy substrates					
Proximal intestine	30.1 ± 1.36^{b}	8.75 ± 0.35^{c}	1.43 ± 0.12^{b}	5.55 ± 0.57^{b}	5.42 ± 0.34^{b}
Liver	8.51 ± 0.40^{c}	13.6 ± 1.55^{b}	0.56 ± 0.05^{c}	1.49 ± 0.13^{c}	1.28 ± 0.08^{c}
Kidney	95.0 ± 2.05^{a}	42.6 ± 3.46^{a}	25.9 ± 1.43^{a}	20.1 ± 1.29^{a}	28.5 ± 1.61^{a}
Skeletal muscle	1.50 ± 0.18^{d}	0.16 ± 0.02^{d}	0.56 ± 0.12^{c}	0.00	0.20 ± 0.02^{d}

Adapted from Table 2.3 Data, expressed as nmol ATP/mg tissue per hour, are mean ± SEM, n = 6. Except for glutamine in the liver and leucine in the proximal intestine, all values obtained in the presence of a mixture of substrates are lower ($P < 0.05$) than the corresponding values obtained in the presence of 5 mM glucose.
a-d: Within a column, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA.

Table 2.6 Comparison of ATP production from a nutrient among zebrafish tissues in the presence of 5 mM glucose or a mixture of energy substrates

Tissue	Glutamate	Glutamine	Leucine	Palmitate	Glucose
In the presence of 5 mM glucose					
Proximal intestine	69.7 ± 2.46^{b}	25.2 ± 2.86^{c}	3.43 ± 0.34^{c}	10.1 ± 0.45^{b}	37.9 ± 2.43^{b}
Liver	29.6 ± 1.32^{c}	33.6 ± 2.66^{b}	24.4 ± 0.77^{b}	2.87 ± 0.08^{c}	14.1 ± 0.85^{c}
Kidney	260 ± 8.08^{a}	78.3 ± 5.72^{a}	33.1 ± 1.72^{a}	37.1 ± 3.49^{a}	63.9 ± 4.01^{a}
Skeletal muscle	3.32 ± 0.15^{d}	2.48 ± 0.28^{d}	1.83 ± 0.33^{d}	1.40 ± 0.18^{d}	0.48 ± 0.05^{d}
In the presence of a mixture of energy substrates					
Proximal intestine	67.2 ± 3.54^{b}	19.1 ± 0.99^{b}	1.24 ± 0.18^{b}	4.48 ± 0.36^{b}	21.5 ± 1.93^{a}
Liver	14.8 ± 1.16^{c}	19.7 ± 1.39^{b}	10.9 ± 1.69^{a}	2.65 ± 0.37^{c}	8.23 ± 0.87^{b}
Kidney	104 ± 6.19^{a}	26.7 ± 1.12^{a}	11.7 ± 1.88^{a}	23.4 ± 2.27^{a}	23.3 ± 2.07^{a}
Skeletal muscle	1.41 ± 0.21^{d}	0.47 ± 0.02^{c}	1.15 ± 0.17^{b}	0.38 ± 0.06^{d}	0.30 ± 0.06^{c}

Adapted from Table 2.4 Data, expressed as nmol ATP/mg tissue per hour, are mean ± SEM, n = 6. Except for glutamate in the proximal intestine and palmitate in the liver, all values obtained in the presence of a mixture of substrates are lower ($P < 0.05$) than the corresponding values obtained in the presence of 5 mM glucose.
a-d: Within a column, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA.

Table 2.7 Tissue weights of a 20-g juvenile hybrid striped bass and a 0.5-g zebrafish and estimated ATP production from the oxidation of a nutrient in the presence of a mixture of energy substrates

Tissue	Tissue weight	Glutamate (2 mM)	Glutamine (2 mM)	Leucine (2 mM)	Palmitate (2 mM)	Glucose (5 mM)
	mg		nmol ATP/h per whole tissue			
			Hybrid striped bass			
Whole intestine	300	9030	2568	420	1665	1626
Liver	400	3404	5440	224	596	512
Kidney	100	9500	4260	2590	2010	2850
Skeletal muscle	8200	12300	1312	4592	0	4428
			Zebrafish			
Whole intestine	9	605	172	11	40	194
Liver	10	148	197	109	27	82
Kidney	2.5	260	67	29	59	58
Skeletal muscle	215	303	101	247	82	65

In the 20-g HSB, the percentages (%) of tissue weights were: skeletal muscle, 41; kidney, 0.50; whole intestine, 1.5; and liver, 2.0. In the 0.5-g zebrafish, the percentages (%) of tissue weights were: skeletal muscle, 43; kidney, 0.50; whole intestine, 1.8; and liver, 2.0.

Table 2.8 Catabolism of [1-^{14}C]leucine by hybrid striped bass and zebrafish tissues in the presence of unlabeled glucose or a mixture of unlabeled substrates

Medium mixture	Oxidative Decarboxylation of Leu (1^{st} collection of $^{14}CO_2$) (A)	α-Ketoisocaproate (KIC) release from the tissue (2^{nd} collection of $^{14}CO_2$) (B)	Net transamination of Leu (A + B)
HSB, nmol CO_2/mg tissue per hour			
		Proximal intestine	
Glucose[1]	0.35 ± 0.02^{b}	0.16 ± 0.007^{c}	0.51 ± 0.03^{a}
Mixture[2]	0.08 ± 0.01^{b}*	0.02 ± 0.001^{c}*	0.10 ± 0.01^{a}*
		Liver	
Glucose[1]	0.34 ± 0.02^{b}	0.07 ± 0.010^{c}	0.41 ± 0.02^{a}
Mixture[2]	0.09 ± 0.01^{b}*	0.03 ± 0.001^{c}*	0.13 ± 0.01^{a}*
		Kidney	
Glucose[1]	1.43 ± 0.09^{b}	1.54 ± 0.10^{b}	2.96 ± 0.12
Mixture[2]	0.99 ± 0.11^{b}*	0.64 ± 0.05^{c}*	1.63 ± 0.17^{a}*
		Skeletal muscle	
Glucose[1]	0.05 ± 0.002^{c}	0.09 ± 0.02^{b}	0.15 ± 0.02^{a}
Mixture[2]	0.02 ± 0.003^{b}*	0.02 ± 0.001^{b}*	0.04 ± 0.004^{a}*
Zebrafish, nmol CO_2/mg tissue per hour			
		Proximal intestine	
Glucose[1]	0.22 ± 0.03^{b}	0.07 ± 0.002^{c}	0.29 ± 0.03^{a}
Mixture[2]	0.13 ± 0.04^{a}*	0.02 ± 0.001^{b}*	0.15 ± 0.04^{a}*
		Liver	
Glucose[1]	2.05 ± 0.15^{b}	0.42 ± 0.05^{c}	2.47 ± 0.19^{a}
Mixture[2]	1.41 ± 0.12^{b}*	0.36 ± 0.04^{c}*	1.77 ± 0.17^{a}*
		Kidney	
Glucose[1]	3.09 ± 0.46^{b}	2.01 ± 0.12^{c}	5.10 ± 0.50^{a}
Mixture[2]	1.85 ± 0.16^{b}*	0.99 ± 0.12^{c}*	2.83 ± 0.27^{a}*
		Skeletal muscle	
Glucose[1]	0.05 ± 0.001^{b}	0.05 ± 0.001^{b}	0.10 ± 0.002^{a}
Mixture[2]	0.03 ± 0.001^{c}*	0.06 ± 0.001^{b}	0.09 ± 0.002^{a}

Values are mean ± SEM, n = 6.
[1] Oxidation of 2 mM [1-^{14}C]leucine in the presence of 5 mM unlabeled glucose.
[2] Oxidation of 2 mM [1-^{14}C]leucine in the presence of a mixture of unlabeled substrates (2 mM glutamate, 2 mM glutamine, 2 mM leucine, 2 mM palmitate, and 5 mM glucose).
a-c: Within a row, means not sharing the same superscript differ ($P < 0.05$), as analyzed by one-way ANOVA.
* $P < 0.05$ vs the value for the oxidation of 2 mM [1-^{14}C]leucine in the presence of 5 mM unlabeled glucose.

Catabolism of [1-^{14}C]Leucine

Data on the catabolism of [1-^{14}C]leucine by tissues of HSB and zebrafish are summarized in Table 2.8. α-Ketoisocaproate (KIC) was a product of leucine transamination in their tissues. Leucine had the highest rate of net transamination in the kidneys of both fish species compared with their other tissues. The rates of net KIC release and the oxidative decarboxylation of leucine were also highest in the kidney. Moreover, the rates of oxidative decarboxylation of leucine in the liver and proximal intestine of both fish species were much greater ($P < 0.05$) than the rates of net KIC release. This was also true for the kidney of zebrafish. The liver had a higher ($P < 0.05$) ratio of the oxidative decarboxylation of leucine to net KIC release than the kidney in both fish species. The rates of net KIC release and the oxidative decarboxylation of leucine by the liver were greater ($P < 0.05$) in zebrafish than in HSB. The rates of oxidative decarboxylation of leucine and net KIC release were low in the skeletal muscle of both species. In all HSB tissues and in the proximal intestine, liver, and kidney tissues of zebrafish, the rates of leucine transamination and oxidative decarboxylation were lower ($P < 0.05$) in the presence of a mixture of energy substrates than the presence of glucose.

Uptake of Amino Acids, Glucose and Palmitate by Tissues from Hybrid Striped Bass

Data on the uptake of different nutrients by HSB tissues are summarized in Table 2.9. In the proximal intestine, kidneys, and skeletal muscle of HSB, the rate of uptake of glutamate was the greatest among all the tested substrates (including glutamine, leucine, palmitate, and glucose), followed by leucine. In the liver of HSB, the rate of uptake of glucose was the greatest among all the tested substrates, followed by glutamate, glutamine and leucine (with a similar rate among the AAs). The proximal intestine had the highest rate of palmitate uptake among the liver, kidney, and skeletal muscle, but skeletal muscle barely

took up palmitate. The rates of uptake of glutamine, leucine, palmitate, and glucose by the proximal intestine, liver, kidney, and skeletal muscle were not affected ($P > 0.05$) by the presence of a mixture of energy substrates compared with glucose alone. This was also true for glutamate, except that its uptake by skeletal muscle was markedly reduced ($P < 0.05$) by the presence of a mixture of energy substrates compared with glucose alone.

Activities of Enzymes

Data on the activities of enzymes in HSB tissues are summarized in Table 2.10. In the proximal intestine, kidneys, and skeletal muscle, the GDH activity was the greatest among all the measured enzymes, followed by GOT. The GS activity was the lowest among all the measured enzymes, followed by CPT-I. In HSB liver tissue, the activities of GOT and GPT were the greatest among all the measured enzymes, followed by GDH. Interestingly, the GS activity was nearly absent from the proximal intestine and skeletal muscle and was very low in the liver and kidney. The activity of K- or L-type phosphate-activated glutaminase in the skeletal muscle was much lower than that in the liver, kidney, and proximal intestine. CPT-I activity was appreciable in the liver, proximal intestine, and kidney, but was barely detected in the skeletal muscle. The activities of key enzymes of glycolysis were relatively high in all the tissues studied.

Table 2.9 Uptake of nutrients by hybrid striped bass tissues in the presence of 5 mM unlabeled glucose or a mixture of unlabeled substrates

Substrate	[U-^{14}C] Glutamate (2 mM)	[U-^{14}C] Glutamine (2 mM)	[U-^{14}C] Leucine (2 mM)	[U-^{14}C] Palmitate (2 mM)	[U-^{14}C] Glucose (5 mM)
			Proximate Intestine		
Glucose[1]	108 ± 21.8^{a}	48.1 ± 6.1^{b}	82.6 ± 7.2^{ab}	50.2 ± 9.2^{b}	59.9 ± 9.2^{b}
Mixture[2]	99.5 ± 8.8^{a}	44.0 ± 6.7^{b}	79.0 ± 16.1^{ab}	49.1 ± 5.2^{b}	56.6 ± 7.2^{b}
			Liver		
Glucose[1]	22.6 ± 3.4^{ab}	19.4 ± 2.6^{b}	23.3 ± 2.4^{ab}	6.50 ± 0.53^{c}	31.2 ± 3.1^{a}
Mixture[2]	19.1 ± 2.2^{b}	21.2 ± 3.1^{b}	24.9 ± 4.6^{ab}	6.66 ± 0.66^{c}	33.5 ± 4.3^{a}
			Kidney		
Glucose[1]	92.7 ± 16.7^{a}	46.5 ± 6.0^{b}	48.2 ± 4.7^{b}	17.0 ± 2.4^{c}	42.6 ± 7.4^{b}
Mixture[2]	82.2 ± 8.5^{a}	41.6 ± 8.8^{b}	43.7 ± 5.6^{b}	15.4 ± 4.4^{c}	42.0 ± 7.0^{b}
			Skeletal muscle		
Glucose[1]	33.2 ± 6.7^{a*}	5.94 ± 1.4^{c}	22.8 ± 4.3^{ab}	0.22 ± 0.08^{c}	19.0 ± 3.5^{b}
Mixture[2]	13.4 ± 1.9^{a}	5.73 ± 1.7^{b}	18.8 ± 3.1^{a}	0.23 ± 0.08^{b}	19.5 ± 3.9^{a}

Data, expressed as pmol/mg tissue per min, are mean ± SEM, n = 6.

[1] Uptake rate of the indicated labeled substrate in the presence of 5 mM unlabeled glucose.

[2] Uptake rate of the indicated labeled substrate in the presence of a mixture of unlabeled substrates (2 mM glutamate, 2 mM glutamine, 2 mM leucine, 2 mM palmitate, and 5 mM glucose).

a-d: Within a row, means not sharing the same superscript differ ($P \leq 0.05$), as analyzed by one-way ANOVA.

* $P < 0.05$ vs the value for the oxidation of substrate alone.

Table 2.10 Activities of enzymes in hybrid striped bass tissues

Enzyme	Liver	Proximal Intestine	Kidney	Skeletal muscle
Glutamate dehydrogenase	446 ± 57.0^{b}	603 ± 43.0^{a}	201 ± 7.10^{c}	17.8 ± 0.47^{d}
Glutamate-pyruvate transaminase	502 ± 30.0^{a}	68.7 ± 4.20^{b}	41.4 ± 3.50^{c}	13.2 ± 1.20^{d}
Glutamate-oxaloacetate transaminase	518 ± 45.0^{a}	352 ± 49.0^{b}	101 ± 13.0^{c}	64.1 ± 5.60^{c}
Branched-chain amino acid transaminase	25.6 ± 0.99^{b}	36.4 ± 5.60^{a}	4.92 ± 1.00^{c}	2.28 ± 0.44^{c}
K-type phosphate-activated glutaminase	3.27 ± 0.33^{a}	3.42 ± 0.18^{a}	1.79 ± 0.27^{b}	0.18 ± 0.03^{c}
L-type phosphate-activated glutaminase	1.23 ± 0.18^{b}	2.67 ± 0.35^{a}	0.89 ± 0.09^{b}	0.23 ± 0.07^{c}
Glutamine synthetase	0.23 ± 0.05^{a}	0.17 ± 0.02^{ab}	0.29 ± 0.05^{a}	0.014 ± 0.001^{b}
Pyruvate kinase	16.9 ± 0.97^{d}	126 ± 9.90^{b}	41.5 ± 4.30^{c}	281 ± 5.10^{a}
Hexokinase	7.41 ± 0.67^{a}	6.47 ± 1.00^{a}	2.04 ± 0.19^{b}	3.41 ± 0.40^{b}
Phosphofructokinase-1	33.2 ± 3.80^{c}	69.6 ± 4.50^{b}	26.2 ± 3.30^{c}	89 ± 5.60^{a}
Carnitine palmitoyltransferase-I	1.70 ± 0.10^{a}	1.78 ± 0.05^{a}	2.03 ± 0.22^{a}	0.17 ± 0.02^{b}

Values, expressed as nmol/mg protein per min, are means and pooled SEM, n = 6.
a-d: Within a row, means not sharing the same superscript letter differ ($P < 0.05$)

Table 2.11 Amounts of metabolites in the homogenate of tissue plus incubation medium after a 2-h period of incubation

Metabolite	0h incubation	2-hour incubation					
		Glucose	Glutamate	Glutamine	Leucine	Palmitate	Mixture
				Liver			
Aspartate	0.14 ± 0.01^{d}	0.18 ± 0.01^{cd}	0.26 ± 0.01^{ab}	0.25 ± 0.02^{ab}	0.22 ± 0.01^{bc}	---	0.30 ± 0.03^{a}
Glutamate	1.42 ± 0.15^{b}	0.71 ± 0.09^{c}	---	3.81 ± 0.29^{a}	1.63 ± 0.11^{b}	---	---
Alanine	6.16 ± 0.29^{a}	4.02 ± 0.25^{b}	4.32 ± 0.30^{b}	3.94 ± 0.32^{b}	4.17 ± 0.22^{b}	---	5.84 ± 0.25^{a}
Ammonia	0.38 ± 0.06^{d}	1.42 ± 0.10^{c}	3.57 ± 0.26^{a}	2.43 ± 0.19^{b}	2.78 ± 0.23^{b}	1.72 ± 0.10^{c}	4.00 ± 0.38^{a}
Lactate	4.48 ± 0.19^{c}	7.55 ± 0.41^{b}	7.88 ± 0.50^{b}	8.15 ± 0.55^{b}	6.12 ± 0.30^{bc}	7.22 ± 0.55^{b}	13.3 ± 1.10^{a}
				Proximal intestine			
Aspartate	0.31 ± 0.04^{b}	1.33 ± 0.15^{a}	1.59 ± 0.12^{a}	1.34 ± 0.11^{a}	1.48 ± 0.15^{a}	---	1.53 ± 0.17^{a}
Glutamate	1.03 ± 0.04^{c}	1.48 ± 0.04^{c}	---	3.22 ± 0.19^{b}	4.53 ± 0.34^{a}	---	---
Alanine	2.14 ± 0.11^{c}	1.75 ± 0.11^{c}	4.97 ± 0.42^{ab}	4.32 ± 0.44^{b}	4.63 ± 0.20^{b}	---	5.74 ± 0.34^{a}
Ammonia	0.57 ± 0.12^{e}	2.22 ± 0.27^{d}	5.43 ± 0.31^{b}	4.62 ± 0.35^{b}	3.65 ± 0.29^{c}	2.47 ± 0.16^{d}	7.05 ± 0.54^{a}
Lactate	9.85 ± 0.91^{c}	29.9 ± 2.76^{a}	29.7 ± 1.11^{a}	19.8 ± 1.82^{b}	21.2 ± 0.70^{b}	22.8 ± 0.98^{b}	28.0 ± 2.39^{a}
				Kidney			
Aspartate	0.16 ± 0.02^{c}	0.24 ± 0.02^{ab}	0.25 ± 0.01^{ab}	0.22 ± 0.01^{b}	0.23 ± 0.02^{ab}	---	0.29 ± 0.01^{a}
Glutamate	1.68 ± 0.09^{b}	1.22 ± 0.10^{b}	---	2.96 ± 0.13^{a}	2.76 ± 0.26^{a}	---	---
Alanine	1.57 ± 0.09^{e}	2.47 ± 0.24^{d}	4.28 ± 0.35^{b}	3.38 ± 0.16^{c}	3.30 ± 0.19^{c}	---	5.64 ± 0.40^{a}
Ammonia	1.43 ± 0.14^{e}	3.87 ± 0.23^{d}	10.5 ± 0.38^{b}	7.68 ± 0.71^{c}	4.72 ± 0.46^{d}	3.13 ± 0.27^{d}	16.4 ± 1.08^{a}
Lactate	9.05 ± 0.30^{d}	20.1 ± 1.28^{b}	24.0 ± 1.18^{ab}	23.8 ± 1.50^{ab}	20.2 ± 0.82^{b}	13.3 ± 0.82^{c}	25.8 ± 2.11^{a}
				Skeletal muscle			
Aspartate	0.10 ± 0.01^{d}	0.16 ± 0.02^{c}	0.25 ± 0.04^{ab}	0.20 ± 0.01^{bc}	0.24 ± 0.02^{ab}	---	0.29 ± 0.02^{a}
Glutamate	0.60 ± 0.06^{b}	0.85 ± 0.06^{b}	---	2.24 ± 0.15^{a}	0.87 ± 0.06^{b}	---	---
Alanine	3.35 ± 0.23^{b}	3.80 ± 0.24^{b}	5.09 ± 0.19^{a}	3.8 ± 0.12^{b}	3.79 ± 0.34^{b}	---	5.18 ± 0.36^{a}
Ammonia	0.23 ± 0.08^{d}	1.25 ± 0.12^{c}	2.18 ± 0.19^{a}	2.08 ± 0.20^{a}	1.83 ± 0.08^{ab}	1.42 ± 0.09^{bc}	2.18 ± 0.21^{a}
Lactate	2.80 ± 0.15^{c}	28.5 ± 2.58^{a}	22.1 ± 1.34^{b}	21.6 ± 1.63^{b}	26.4 ± 1.11^{ab}	23.9 ± 1.23^{ab}	24.7 ± 0.79^{ab}

Values, expressed as nmol/mg tissue per hour, are means and SEM, n = 6.
---: not determined.
a-d: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).
Mixture contained 2 mM of glutamate, glutamine and leucine and 5 mM glucose.

Discussion

Animals exhibit tissue and species differences in nutrient metabolism (Wu 2017). Glutamate and glutamine are extensively oxidized by the small intestine of pigs and rats to generate a large amount of ATP (Wu 1998, 2010). In contrast, both glucose and fatty acids are the major metabolic fuels for the kidneys and skeletal muscle of mammals, and fatty acids are the primary source of energy in the liver of mammals (Jobgen et al. 2006). As noted previously, both omnivorous and carnivorous fish have a lower capacity to utilize dietary starch than omnivorous mammals and birds. There are reports that the skeletal muscle of an Antarctic teleost, *Gobionotothm gibberifrons*, actively oxidizes long-chain fatty acids to CO_2 (Sidell et al. 1995), but the hepatocytes of fed rainbow trout have a limited ability to oxidize palmitate to CO_2 (French et al. 1981). Polakof et al. (2010) have shown that the intestine of rainbow trout contains enzymes to metabolize glucose into lactate. Presently, major sources of energy substrates for specific tissues in fish are unknown.

The use of radiolabeled nutrients provides an approach to identifying and quantifying their metabolic pathways in animal tissues (Wu 2013a). This study determined, for the first time to our knowledge, the rates of the oxidation of glutamate, glutamine, leucine, glucose, and palmitate individually or as a mixture of substrates in the proximal intestine, liver, kidney, and skeletal muscle of fish. Dietary glutamate, glutamine, and leucine are abundant in proteins of animal and plant origins, such as fish meal, poultry by-product meal and soybean meal, which are widely used as protein sources for fish feeds (Li et al. 2011). Moreover, it is well known that glutamate, glutamine and their metabolites participate in multiple metabolic pathways, such as glutaminolysis, transamination, and the Krebs cycle (Wu 2017). Glutamine and glutamate are regulators of gene expression and cell signaling in mammals (Wu 2010). Relatively high activities of BCAT make the mammalian skeletal muscle the major site for initiating BCAA transamination in the body. Hence, a large amount of leucine (one of the

BCAAs) is degraded by mammalian skeletal muscle to generate KIC. Based on the published studies involving mammals (Wu 2013a), glutamate, glutamine, and leucine were chosen for the present investigation. For comparison, glucose and palmitate were used as the representatives for carbohydrates and lipids, respectively.

Glutamate and Glutamine Oxidation

The rate of CO_2 production from glutamate in the presence of a mixture of energy substrates varied greatly among tissues of fish. This AA was the most oxidative substrate in the proximal intestine, kidney, and skeletal muscle, and the second most oxidative substrate (after glutamine) in the liver of both HSB and zebrafish (Tables 2.1 and 2.2). The proximal intestine, kidneys, skeletal muscle, and liver together comprised 45% and 47% of the body weight in the juvenile HSB and young adult zebrafish, respectively. Our data indicate a quantitatively important role of glutamate oxidation in producing ATP in fish tissues. To generate ATP, the carbon backbone of glutamate is converted to α-ketoglutarate by GDH, GPT, or GOP. Both GDH and glutamate transaminases were found at much higher activities than fructose bisphosphatase in the livers of rainbow trout (French et al. 1981) and the sea bass (Enes et al. 2006). Moreover, Tng et al. (2008) reported that GDH activity in the liver and intestine of juvenile *O. marmorata* was increased by feeding. Likewise, GDH activity is highest in the kidneys of fish among digestive tissues and skeletal muscle (Christiansen and Klungsøyr 1987). However, in HSB, GDH activity was much greater in the proximal intestine than in the kidneys (Table 2.10), which was consistent with the metabolic data (Table 2.1 and 2.11). This is contrast to mammals, whose intestines have little GDH activity (Wu 2013a). Many tissues (including the liver, intestine, kidney and muscle) of teleost and non-teleost fish possess a series of enzymes (including malic enzyme) to convert glutamate-derived α-KG into pyruvate (Chamberlin et al. 1991), which is subsequently oxidized to CO_2

via pyruvate dehydrogenase and the Krebs cycle (Wu 2017). This is also true for the HSB (Table 2.10). Therefore, a higher oxidative rate of glutamate over other nutrients in fish intestine, kidneys, and skeletal muscle was due to higher activities of GDH, GPT, and GOT than the enzymes that degrade glutamine, glucose, and palmitate (Table 2.10). Indeed, in HSB, the activity of K- or L-type phosphate-activated glutaminase was less than 1% of GDH activity in the proximal intestine and liver and less than 1.5% of GDH activity in the kidney and skeletal muscle. As a major energy substrate, glutamate is crucial for fish growth, development, and health. This finding supports the use of glutamate to improve intestinal morphology, function, and whole-body growth in rainbow trout that are fed a soybean meal-based diet (Yoshida et al. 2016).

Glutamine was readily oxidized in the liver, proximal intestine, and kidneys of both HSB (Table 2.1) and zebrafish (Table 2.2), despite a lower rate of CO_2 production from glutamine than glutamate by the proximal intestine and kidneys in the presence of a mixture of energy substrates as noted previously. Oxidation of glutamine also occurred in the skeletal muscle of HSB (Table 2.1) and zebrafish, as reported for rat and chicken skeletal muscles (Wu et al. 1991). Interestingly, we found that there was a very low rate of glutamine oxidation in fish skeletal muscle incubated with a mixture of energy substrates (Tables 2.1 and 2.2). This contrasts with the finding that mitochondria isolated from the lateral red muscle of teleost (*Salvelinus namaycush*) and nonteleost fish (*Amia calva*) fish actively oxidize glutamine (10 mM in the incubation medium) to CO_2 (Chamberlin et al. 1991). Of note, glutamine was the most important source of ATP in the liver of both HSB and zebrafish (Table 2.3 and 2.4). This is consistent with the suggestion of Caballero-Solares (2015) that the liver of fish oxidizes glutamine preferentially over other dietary AAs. Thus, glutamine can be actively taken up by the hepatocytes of fish. In certain mammalian cells (e.g., tumors),

glutamine can contribute 30-50% of energy in the presence of physiological levels of glucose (Zielke et al. 1984).

Phosphate-activated glutaminase (a mitochondrial enzyme) plays a major role in initiating glutamine degradation in most mammalian tissues, including the small intestine, kidneys and skeletal muscle (Wu 2013a). This enzyme converts glutamine into glutamate and ammonia, and its activity is relatively high in the kidneys but very low in the white muscle of lake char fish (Chamberlin et al. 1991). There are also reports that ammonia is produced mainly in the liver mitochondrial matrix of ammoniotelic fishes (Ip and Chew 2010) and that glutamine degradation via glutaminase can account for 85% of the total ammonia excreted from some fish (Campbell et al. 1983). Our finding that a greater amount of CO_2 was produced from glutamate than glutamine in the intestine (Table 2.1) may be explained by greater activities of GDH and glutamate transaminases in the intestine (Table 2.10). This is in contrast to mammals, in which GDH activity is high in the liver but nearly absent from the intestine (Bush et al. 2002). In addition, because the rate of oxidation of glutamate by the small intestine was two to four times greater than that of glutamine in HSB and zebrafish, it is possible that inhibition of K-type phosphate-activated glutaminase by a high concentration of intracellular glutamate (Wu 2013a) limits the intestinal catabolism of glutamine. More research is warranted to test this hypothesis.

Fish tissues may interconvert glutamate and glutamine. When an incubation medium contained both of these AAs, the intracellular specific radioactivity of [U-^{14}C]glutamate or [U-^{14}C]glutamine in a tissue may be affected by the presence of extracellular unlabeled glutamine or glutamate, respectively. We found that under the experimental conditions used (e.g., 2 mM glutamate and 2 mM glutamine in the incubation medium), the intracellular specific radioactivity of [U-^{14}C]glutamate [measured as previously described (Wu et al. 1991)] in the presence of extracellular unlabeled glutamine was not affected in skeletal

muscle, was about 6% lower in the small intestine and liver, and was about 10% lower in the kidneys for both zebrafish and HSB when compared with the absence of glutamine. We also observed that the intracellular specific radioactivity of [U-^{14}C]glutamine in the presence of extracellular unlabeled glutamate was not affected in the kidneys and small intestine and was about 7% lower in the liver and skeletal muscle for both zebrafish and HSB when compared with the absence of glutamate. Because there was only a small change in the intracellular specific radioactivity of [U-^{14}C]glutamate or [U-^{14}C]glutamine in the fish tissues, we concluded that the presence of both unlabeled glutamate and glutamine (2 mM each) in the incubation medium did not substantially underestimate the rates of oxidation of these two AAs to CO_2.

Leucine Oxidation

Both [1-^{14}C]leucine and [U-^{14}C]leucine have been employed to determine the metabolic pattern of leucine in cells and tissues of terrestrial animals (Wu and Thompson 1987; Lei et al. 2012, 2013). Much is known about interorgan catabolism of BCAAs in mammals (Wu 2013a). In their extrahepatic tissues such as the small intestine and skeletal muscle, BCAAs undergo active transamination with α-ketoglutarate to form branched-chain α-ketoacids (BCKAs) and glutamate. The small intestine of pigs extracts 20-40% of dietary BCAAs in the first pass, thereby affecting the availability of these AAs for utilization by other organs (Hou et al. 2015, 2016b). In avian and mammalian skeletal muscles, BCAAs are used to synthesize glutamine and alanine, and these metabolic pathways are of nutritional and physiological significance (Wu 2013a). In both the small intestine and skeletal muscle, the decarboxylation of BCKAs is limited due to a low activity of BCKA dehydrogenase; therefore, most of the BCKAs are released into the extracellular space (Wu 2013a). In mammals, the liver and the kidneys play a major role in oxidizing BCKAs released from

other tissues. Due to a low activity of hepatic BCAT, the mammalian liver has a limited capacity for degrading BCAAs (including leucine) to CO_2 compared with the kidneys (Dawson et al. 1967; Wijayasinghe et al. 1983). Likewise, BCAT activity in the liver of lake trout is much lower than that in their kidneys and skeletal muscle (Hughes et al. 1983). Similar results were reported for the homogenates of tissues (e.g., kidney and skeletal muscle) from rainbow trout (Teigland and Klungsøyr 1983). In contrast, in HSB, BCAT activity was high in the liver, proximal intestine, and kidney while being relatively low in the skeletal muscle (Table 2.10). Among the HSB and zebrafish tissues examined, the rates of net leucine transamination were the highest in the kidney and the second highest in the liver, but lowest in the skeletal muscle (Table 2.8). A higher rate of leucine transamination in the kidney than in the liver may be explained by a higher rate of leucine uptake by the kidney (Table 2.9). In all incubated tissues except for the zebrafish muscle, the rates of net leucine transamination were markedly inhibited by the presence of a mixture of energy substrates, which likely have a sparing effect on BCAA utilization by fish. Our finding that about 82%, 70%, 50%, and 30% of the KIC produced from leucine was decarboxylated by the liver, proximal intestine, kidney, and skeletal muscle of HSB, respectively (Table 2.8), indicates a low activity of BCKA dehydrogenase in the muscle. Based on the rates of CO_2 production from ^{14}C-labeled substrates, our results showed that: (1) leucine oxidation produced more ATP in the kidneys than in other tissues of HSB and zebrafish; (2) leucine was a minor metabolic fuel in the intestine, liver, and kidney of HSB and zebrafish, as well as the skeletal muscle of HSB; and (3) leucine could contribute to about one-third of ATP production in zebrafish skeletal muscle where palmitate oxidation was not detectable in the presence of a mixture of energy substrate (Table 2.7). This illustrates a difference in AA metabolism between these two species.

Fatty Acid and Glucose in Tissues from Hybrid Striped Bass

In mammals, the liver and skeletal muscle are two major tissues for the oxidation of long-chain fatty acids (Jobgen et al. 2006). This, however, is not true for HSB (Table 2.1). There was little oxidation of palmitate in the skeletal muscle of HSB (Table 2.1) due to the near absence of palmitate uptake (Table 2.9) and CPT-I activity (Table 2.10). In the presence of a mixture of energy substrates, glucose oxidation in the skeletal muscle of HSB was limited (Table 2.1) possibly by the production of acetyl-CoA (an inhibitor of pyruvate dehydrogenase) from AAs rather than by the activities of enzymes that convert glucose into pyruvate. These findings may partly explain, why HSB cannot tolerate a high intake (e.g., > 30%) of dietary starch (Stone 2003) and can guide the feeding of these fish.

Conclusion

Glutamate and glutamine were more actively oxidized in the proximal intestine, liver, and kidney of fish over the oxidation of glucose and palmitate. Glutamate provided more energy than glutamine, glucose, and palmitate in all the tissues except in the liver, where glutamine served as the main metabolic fuel. In the skeletal muscle of HSB and zebrafish, glutamate was the preferred nutrient to generate ATP, followed by leucine and glucose in HSB or by leucine and glutamine in zebrafish. Together, glutamate plus glutamine plus leucine contributed to about 80% of ATP production in fish tissues. Fish tissues had high activities of GDH, GOT and GPT, as well as high rates of glutamate uptake by, which provided a biochemical basis for their extensive catabolism of glutamate. Therefore, we suggest that AAs (primarily glutamate and glutamine) are the major metabolic fuels for the proximal intestine, liver, kidney, and skeletal muscle of HSB and zebrafish. These findings not only help show why fish have particularly high requirements for dietary protein, but also

have important implications for improving fish diets to provide optimal levels of protein, carbohydrates, and lipids.

CHAPTER III

DEVELOPMENT OF FISHMEAL-FREE FEED FOR YUEHAI FISH FEED COMPANY

REPLACEMENT OF FISHMEAL-FREE FEED WITH POULTRY BY-PRODUCT MEAL AND SOYBEAN PROTEIN CONCENTRATE FOR JUVENILE HYBRID STRIPED BASS

Synopsis

Poultry by-product meal (PBM) and soybean protein concentrate (SPC) contained high levels of proteins in which glutamate and glutamine are highly abundant. This study investigated the potential of using PBM, SPC and crystalline amino acids (AAs) to replace fishmeal (FM) in the diets of juvenile hybrid striped bass (HSB). The major measured variables included growth performance, muscle protein synthesis rate, proximate anlaysis of whole body, and six-hour-postprandial plasma AA concentrations. Six diets containing about 40% crude protein and about 11% crude lipids were formulated, in which FM protein was replaced by the mixture of half PBM and half SPC at 0% (60% FM in diet, no FM replacement) as a control group, 15% (45% FM in diet), 30% (30% FM in diet), 45% (15% FM in diet), 57% (3% FM in diet), and 60% (0% FM in diet), respectively. This represented 0%, 25%, 50%, 75%, 95% and 100% FM replacement. Each test diet was supplemented with a mixture of four AAs (lysine, methionine, threonine, and taurine) to ensure it contained similar levels of essential AAs and taurine to those in the 60% FM diet. Each diet was randomly assigned to triplicate tanks of 12 three-month-old HSB (initial weight 5.5 ± 0.1 gram). The fish were maintained in a recycling aquaculture system in which water was circulated through mechanical and biological filters and changed regularly (30-50% daily). Air was supplied through air stones connected to an air pump. The dissolved oxygen was maintained above 6 mg/L, salinity at 1-1.5 mg/L, and photoperiod at 14 hours per day. Fish were fed four times daily for four weeks at 7-8% of their body weights, which were measured

weekly. The growth performance parameters (weight gain, specific growth rate, feed conversion rate, and protein efficiency ratio) showed a similar trend: 1) fish from the 45% FM and 30% FM groups grew better than those from the 60% FM group; 2) the growth performance of fish in the 15% FM and 3% FM groups were not different from those of fish in the 60% FM group; and 3) fish in the 0% FM group grew significantly worse than those in the 60% FM group. The hepatosomatic indices of fish in the 3% FM and 0% FM groups were significantly lower than that of other groups. The visceral fat index decreased as the percentage of FM decreased, but only the visceral fat index of fish in the 0% FM group was significantly lower than those of other groups. Crude lipid content in the whole body of fish in the 0% FM group was significantly lower than other groups. Moisture, crude protein, and ash contents of the whole body of fish in all groups did not differ. Rates of protein synthesis in skeletal muscle were not different. These results indicate that a mixture of protein from PBM and SPC with AA supplements could effectively replace up to 75% of FM protein in the HSB diet without adverse effects on growth performance.

Introduction

Fish meal (FM) has been the optimal protein ingredient for all fish species, especially carnivorous fish, because of its high digestibility, palatability, and favorable amino acid (AA) profile (Hardy 2010). The FM-based diet is now widely used in the aquaculture industry. However, owing to the rapidly expanding aquaculture and constant annual global production of FM, FM price increases in a fluctuating way. To solve this problem, aquaculture researchers have prioritized to find an alternative to FM for all fish species.

Because of their high protein and high essential AA (EAA) content, poultry by-product meal (PBM) and soybean protein concentrate (SPC) are two major ingredients to replace FM due to their availability, sustainability, and consistent market price. The PBM has

been tested to replace FM in the diets of many species (Nengas et al. 1999; Rawles et al. 2009; Hill et al. 2019). However, fish exhibited a reduction in growth performance when a high level of PBM was included in their diets (Rawles et al. 2009). Supplementing those diets with the first limiting AA might ameliorate suboptimal performance (Gaylord and Rawles 2007). One study reported that Nile tilapia fed diets only containing meat & bone meal or a high ratio of meat & bone meal to PBM (3:1 vs 2:3) with supplemental lysine outperformed the fish fed an FM-based diet (Davies et al. 1989). Rawles et al. (2009) reported that petfood-grade PBM could replace all FM in commercial diets for HSB with a mean body weight of 76 g. However, no such information is available for juvenile HSB, which could be more sensitive to FM replacement due to their fast growth. Because lysine, methionine and threonine are usually limiting AAs in plant- and poultry byproduct-based diets (Rawles et al. 2006; U.S. Soybean Export Councel 2008) and because taurine content in PBM is only half of that in FM and SPC is devoid of taurine (Li et al. 2009b), these four AAs were chosen to be supplemented along with SPC and poultry byproducts to FM replacement diets.

Materials and Methods

Preparation of Experimental Diets

Poultry by-product meal (PBM) and soybean protein concentrate (SPC) were used to replace total or partial FM in HSB diets, and crystalline AAs were added to balance the AA content among all groups. Diets were formulated to contain ~40% crude protein, 11% lipids, and ~3.39 kcal of digestible energy (DE) g^{-1}. Digestible energy was calculated based on the physiological fuel values of 4, 4, and 9 kcal/g for carbohydrates, protein, and lipids, respectively. All diets had the same level of dextrinized starch, carboxymethyl cellulose, and fat. The proximate composition and AA profile of the protein ingredients of the experimental dies is presented in Table 3.1. The formulation is presented in Table 3.2. The content of

amino acids of each test diet was calculated based on Table 3.1 and 3.2, as shown in Table 3.3. The nutrition value of all diets met or exceeded the HSB nutrient requirements recommended by the NRC (2011). Dry ingredients were mixed in air-inflated and sealed bags. The dry mixture was then blended with oil and adequate water using a mixer. The dough was pelleted by a professional meat grinder with a 1/8-inch plate. The pellets were dried using forced air at room temperature (25°C) overnight and stored in sealed bags at -20°C until use.

Fish and Feeding

The feeding trial took place at the vivarium of Kleberg Center Building, Texas A&M University and ended after the fourth week. Juvenile HSB (*Morone saxatilis* ♀ × *Morone chrysops* ♂) were obtained from Keo Fish Farm (Keo, Arkansas, USA). HSB were maintained in a water cycling system at a temperature of 25 ± 1℃ for at least a week to acclimate to local conditions and fed with commercial fish feed. Water was circulated through mechanical and biological filters and changed regularly (30-50% daily). System water was prepared by mixing distilled water from the building with Instant Ocean sea salt at the salinity of 1-1.5 mg/l. Air was supplied through air stones connected to an air pump, and photoperiod was maintained at 14 h per day. Dissolved oxygen was maintained above 6 mg/l in each tank. The pH, ammonia, nitrite, and nitrate were monitored weekly and remained within acceptable limits.

Twelve fish (5.5 ± 0.1 g) were randomly assigned to each tank. Each diet was fed to triplicate tanks of fish. Except the group that was fed the 0% FM diet, 12 fish of each tank were fed the same amount of dry matter feed that was calculated based on the average of body weight of the control group bulk-weighed every week. The ration was fed at 8%, 7.5%, 7.5%, and 7% of the body weight of the control group for the first, second, third, and fourth

weeks respectively. However, due to the poor palatability of the 0% FM diet, the group that was fed with the 0% FM diet could not consume as much as other groups. Therefore, this group was fed to apparent satiation and the food intake was recorded separately. All groups were fed four times a day at 09:00, 13:00, 17:00, and 21:00 in order to maximize the anabolic efficacy of each diet.

Sample Collection and Analysis

At the end of week 4, fish were bulk-weighed and not fed overnight. The next morning, fish were fed to apparent satiation. Two fish per tank were sampled for blood collection from their tails using a heparinized syringe at six hours postprandial. Their individual body weights, liver weights, and visceral fat weights were also recorded. Blood was quickly centrifuged at 3000× g for two minutes at 4°C. Supernatant plasma was collected and stored at -80°C for later AA analysis. Plasma AAs were quantified using HPLC methods involving precolumn derivatization with ophthaldialdehyde as previously described (Dai et al. 2014a).

Six fish per treatment were sampled for whole body proximate analysis including contents of crude protein, lipid, moisture, and ash (AOAC, 1990). Another six fish per treatment were sampled six hours after feeding for protein synthesis studies. Fish had the highest total amount of plasma AAs around four to eight hours postprandial, which was the reason we sampled them six hours after feeding to satiation. Fish were euthanized via MS-222, 400 mg/l before sampling. All experimental procedures were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

In vitro Protein Synthesis by Muscle of Hybrid Striped Bass

Protein synthesis rates of HSB intestine and skeletal muscle were determined as previously described (Kong et al. 2012). Briefly, a customized DMEM medium (formulation Table B.1 is in Appendix B) including 5 mM glucose, 0.1 nM insulin, 1 mM L-[ring-2, 6-^{3}H(N)] phenylalanine, and other basal amino acids was gassed with 95% O_2 and 5% CO_2 for five minutes. A 20-40 mg fresh and thin-cut tissue slice was incubated in 1 ml of the special DMEM medium at 26°C with 95% O_2 and 5% CO_2 gas for two hours. The tissue was rinsed with ice-cold phosphorous-buffered saline (pH 7.4) three times after incubation then homogenized with 2 ml of 2% TCA on the ice. The homogenate was centrifuged at 600 × g for five minutes at 4°C. The pellet was washed twice with 2% TCA and then dissolved in 0.5 ml of 1 N NaOH at 37°C until full dissolution. The solution with dissolved tissue was used for ^{3}H counting.

Calculation and Statistical Analysis

Data were analyzed by one-way analysis of variance and the Student-Newman-Keuls (SNK) multiple comparison test (Assaad et al. 2014). Log transformation of variables was performed when the variances of data were not homogenous among treatment groups, as assessed by the Levene's test. Probability values (*P*-values) less than 0.05 were taken to indicate statistical significance.

Table 3.1 Proximate composition and amino acid profile of the protein ingredients of test diets fed to hybrid striped bass

Component	Menhaden fish meal[a]	Poultry by-product meal[b]	Soybean protein concentrate[c]
Proximate composition (%, as fed)			
Dry matter (%)	92.0	95.8	94.3
Crude protein (%)	69.3	68.0	72.2
Crude lipid (%)	9.0	15.7	1.0
Amino acid (%, as fed)			
Alanine	5.07	4.91	2.38
Arginine	4.85	4.63	5.01
Asparagine	2.92	2.73	ND
Aspartic acid	4.35	4.10	6.45[d]
Cystine	0.67	1.05	1.03
Glutamine	3.94	3.54	ND
Glutamate	6.01	4.89	13.6[e]
Glycine	6.58	9.41	2.39
Histidine	1.51	1.30	1.79
Hydroxyproline	1.86	3.31	0.00
Isoleucine	3.26	2.32	3.15
Leucine	5.24	4.21	5.38
Phenylalanine	2.78	2.36	3.39
Proline	4.25	6.71	2.93
Serine	2.81	2.67	3.00
Tryptophan	0.70	0.49	0.77
Threonine	4.12	2.85	2.74
Tyrosine	2.37	1.84	2.47
Valine	3.81	2.88	3.36
Lysine	4.45	3.51	3.37
Methionine	1.94	1.48	0.78
Taurine	0.84	0.46	0.00

a. SeaLac fish meal from Omega Protein, Reedville, VA. Values are from Li et al. 2009b
b. Obtained from National Renderers Association, Alexandria, VA. Values are from Li et al. 2009b
c. Profine VF, Solae Company, St. Louis, MO. Values are from USDA, 2018
d. Sum of aspartate + asparagine
e. Sum of glutamate + glutamine
ND: not determined.

Table 3.2 Formulation of experimental diets in the feeding trial

Ingredient (% dry weight)	60%FM	45%FM	30%FM	15%FM	3%FM	0%FM
Menhaden fishmeal[a]	60.0	45.0	30.0	15.0	3.00	0.00
Soy protein concentrate[b]	0.00	7.50	15.0	22.5	28.5	30.0
Poultry by-product meal[c]	0.00	7.50	15.0	22.5	28.5	30.0
Fish oil	0.70	2.00	3.30	4.70	5.70	6.00
Soybean oil	0.30	0.20	0.20	0.10	0.00	0.00
Poultry fat	4.70	3.50	2.40	1.20	0.20	0.00
Taurine	0.00	0.10	0.20	0.30	0.40	0.40
Lysine	0.00	0.20	0.40	0.50	0.70	0.70
Methionine	0.00	0.10	0.30	0.40	0.50	0.50
Threonine	0.00	0.20	0.40	0.70	0.80	0.90
Dextrinized starch	20.0	19.6	19.2	18.8	18.5	18.4
Vitamin premix[d]	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[e]	1.00	1.00	1.00	1.00	1.00	1.00
Cellulose	8.30	8.00	7.70	7.40	7.20	7.10
Carboxymethyl cellulose	2.30	2.30	2.30	2.30	2.30	2.30
$Ca(H_2PO_4)_2 \cdot H_2O$	0.80	0.80	0.80	0.80	0.80	0.80
K_2HPO_4	0.50	0.50	0.50	0.50	0.50	0.50
$CaHPO_4$	0.50	0.50	0.50	0.50	0.50	0.50
Crude protein[f]	38.3	38.6	38.8	39.3	39.8	40.2
Crude lipid[f]	11.0	11.0	11.0	11.0	11.0	11.0

a. SeaLac fish meal from Omega Protein, Reedville, VA.
b. Profine VF, Solae Company, St. Louis, MO.
c. Obtained from National Renderers Association (Alexandria, VA, USA).
d. Providing the following (mg/kg of the complete diet): vitamin A acetate, 23.06; cholecalciferol, 20.24; DL-α-tocopheryl acetate, 200; menadione, 12; ascorbic acid, 300; DL-calcium pantothenate, 109; myo-inositol, 150; niacin, 140; pyridoxine-HCl, 30.38; riboflavin, 30; thiamine mononitrate, 32.6; biotin, 1.5; folic acid, 6; vitamin B_{12}, 0.2; and carnitine, 0.08.
e. Providing the following (mg/kg of the complete diet): chromium(III) chloride, 7.3; $CuSO_4.5H_2O$, 35; $FeSO_4.7H_2O$, 498; $MnSO_4.H_2O$, 82; Na_2SeO_3, 3; $ZnSO_4.7H_2O$, 258; sodium molybdate, 0.26; sodium fluoride, 1.3; $CoCl.6H_2O$, 5.2; KI, 7.8; and NiCl, 2.2.
f. Calculated from the ingredient nutrition information (See Table 5.1).

Table 3.3 Calculated content of amino acids in test diets fed to hybrid striped bass

Amino acids	60%FM	45%FM	30%FM	15%FM	3%FM	0%FM
Alanine	3.04	2.81	2.61	2.40	2.23	2.19
Arginine	2.91	2.88	2.90	2.90	2.89	2.89
Asp+Asn	4.36	4.23	4.17	4.08	4.00	3.98
Cystine	0.40	0.45	0.51	0.57	0.61	0.62
Glycine	3.95	3.82	3.74	3.64	3.56	3.54
Glu+Gln	5.97	6.07	6.29	6.45	6.58	6.61
Histidine	0.91	0.90	0.92	0.92	0.93	0.93
Hydroxyproline	1.12	1.08	1.05	1.02	1.00	0.99
Isoleucine	1.96	1.86	1.80	1.72	1.66	1.64
Leucine	3.14	3.05	3.01	2.94	2.89	2.88
Phenylalanine	1.67	1.67	1.70	1.71	1.72	1.73
Proline	2.55	2.61	2.72	2.81	2.87	2.89
Serine	1.69	1.68	1.69	1.70	1.70	1.70
Tryptophan	0.42	0.41	0.40	0.39	0.38	0.38
Threonine	2.47	2.50	2.50	2.50	2.50	2.50
Tyrosine	1.42	1.38	1.36	1.33	1.30	1.29
Valine	2.29	2.17	2.08	1.98	1.89	1.87
Lysine	2.67	2.70	2.70	2.70	2.70	2.70
Methionine	1.16	1.20	1.20	1.20	1.20	1.20
Taurine	0.50	0.51	0.52	0.53	0.50	0.54

Note: Data are expressed as g/100 g dry matter. All values were calculated from the ingredient nutrition information (Table 3.1) and Formulation of the test diets (Table 3.2)

Results

The growth data over four weeks and the growth performance data after 4 weeks are presented in Table 3.4 and Table 3.5, respectively. The body weight of fish that were fed a diet without FM was lowest from the first week to the end, whereas the 45% FM and 30% FM groups showed the highest body weight from the first week until the end. The body weight of the 60% FM group grew slower than those of the 45% FM and 30% FM groups, but did not differ those of the 15% FM and 3% FM groups. After 4 weeks, the 45% FM and 30% FM groups gained more weight than other groups, and the 3% FM and 60% FM groups showed no difference in weight gain in four weeks although the 60% FM group had about 30% more weight gain than the 3% FM group. Specific growth rate also had a similar pattern

to weight gain. The feed conversion ratios (FCRs) and protein efficiency ratios (PERs) of the diets of the 45% FM and 30% FM groups showed the highest efficiency. The hepatosomatic index of the 3% FM and 0% FM groups was lower than that of other groups. The 0% FM group had the lowest visceral fat index, while that of other groups did not differ.

Data on the proximate composition of the HSB that were fed diets with different levels of protein are shown in Table 3.6. Moisture, crude protein, and ash levels of HSB were not affected by the FM levels in the diet within four weeks. However, the crude lipid level of the 0% FM group was lower than those of the 60% FM, 45% FM, and 30% FM groups.

Data on *in vitro* protein synthesis rate of the skeletal muscle of HSB that were fed a diet with different levels of FM are presented in Figure 3.1. There was no difference in protein synthesis rate among all groups. The rates of all groups were about 250 picomole/mg fresh tissue per hour.

Data on free AAs of plasma of HSB 6 hours after feeding are in Table 3.7. The concentration of most FAAs among all groups did not differ except for aspartate, asparagine, serine, glutamine, histidine, citrulline, and tryptophan. Plasma aspartate, asparagine, serine, glutamine, and tryptophan concentrations increased as more FM was replaced by PBM and SPC in the diets. Plasma histidine and citrulline had a higher concentration in HSB that were fed a diet with higher FM, and vice versa. The total amount of AAs increased as more FM was replaced by PBM and SPC in the diets, but only differ in the 0%FM group. The total amount of aspatate + glutamate + glutamine + alanine in the plasma increased as more FM was replaced by SPC and PBM in the diets.

Table 3.4 Growth rates of hybrid striped bass that were fed the experimental diets for four weeks

	60%FM	**45%FM**	**30%FM**	**15%FM**	**3%FM**	**0%FM**	**P-value**	**Pooled SEM**
Day0	5.49	5.51	5.49	5.51	5.53	5.52	0.45	0.02
Day7	9.11^{ab}	9.59^{a}	9.67^{a}	9.22^{ab}	8.91^{ab}	8.66^{b}	0.01	0.26
Day14	12.2^{b}	13.6^{a}	13.5^{a}	12.5^{b}	11.4^{b}	11.5^{b}	0.00	0.41
Day21	16.4^{c}	18.7^{a}	17.7^{b}	16.6^{c}	15.6^{c}	14.4^{d}	0.00	0.38
Day28	22.1^{b}	24.8^{a}	23.8^{a}	21.6^{b}	20.7^{b}	17.9^{c}	0.00	0.63

Values, expressed as g/fish, are means and pooled SEM, n = 3 per treatment group.
Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the SNK test. Growth rate graph (Figure B.1) is available in Appendix B

Table 3.5 Growth performance of hybrid striped bass that were fed the experimental diets

	60%FM	45%FM	30%FM	15%FM	3%FM	0%FM	P-value	Pooled SEM
Final weight (g/fish)	22.1^{b}	24.8^{a}	23.8^{a}	21.6^{b}	20.7^{b}	17.9^{c}	0.00	0.63
Weight gain[a] (%)	303^{b}	349^{a}	334^{a}	292^{b}	274^{b}	225^{c}	0.00	11.2
Survival (%)	100	100	100	100	100	100	1.00	0.00
Feed intake (g DM/fish)	18.5	18.5	18.5	18.5	18.5	17.0	0.07	0.34
SGR[b]	4.97^{b}	5.37^{a}	5.25^{a}	4.88^{b}	4.71^{b}	4.20^{c}	0.00	0.11
FCR[c]	1.18^{b}	1.01^{c}	1.06^{c}	1.21^{b}	1.29^{ab}	1.37^{a}	0.00	0.05
PER[d]	2.13^{b}	2.47^{a}	2.35^{a}	2.07^{b}	1.94^{bc}	1.83^{c}	0.00	0.08
HSI[e]	2.27^{a}	2.23^{a}	2.28^{a}	2.25^{a}	1.70^{b}	1.74^{b}	0.00	0.13
VFI[f]	6.82^{a}	6.59^{a}	5.74^{ab}	5.71^{ab}	5.42^{ab}	4.66^{b}	0.00	0.53

a. Weight gain (%) = 100 × [(final weight (g) – initial weight (g)) / initial weight (g)].
b. Specific growth rate (% body weight/d) = 100 × [(ln average final weight – ln average initial weight)]/days
c. Feed conversion ratio = total dry matter diet fed (g) / total wet weight gain (g).
d. Protein efficiency ratio = protein gain (g)/protein fed (g).
e. Hepatosomatic index = 100 × [liver weight (g) / fish body weight (g)]
f. Visceral fat index (VFI) = 100 × [visceral fat weight (g)/ fish body weight (g)]

Values are means and pooled SEM, n = 3 per treatment group for all variables except HSI and VFI which has n = 6.
Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the SNK test.

Table 3.6 Composition of hybrid striped bass that were fed diets containing different levels of fish meal

%	60%FM	45%FM	30%FM	15%FM	3%FM	0%FM	P-value	Pooled SEM
Moisture	68.4	68.7	67.0	67.8	68.0	68.3	0.65	1.06
Crude protein	17.2	17.3	17.5	17.6	17.8	17.9	0.11	0.38
Crude lipid	11.5^{ab}	11.6^{ab}	11.4^{a}	11.5^{ab}	10.8^{ab}	10.0^{b}	0.04	0.55
Ash	4.33	4.31	4.48	4.15	4.37	4.42	0.39	0.15

Values are means and pooled SEM, n = 6 per treatment group.
a-b: Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the SNK test.

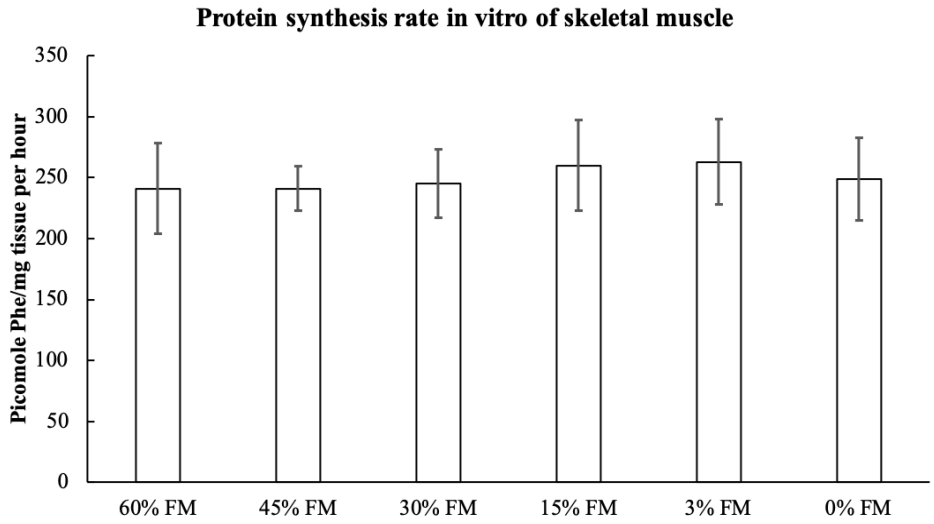

Figure 3.1 Protein synthesis rate in vitro of skeletal muscle of hybrid striped bass that were fed the test diets. Values, expressed as picomole/mg tissue per hour, are means and pooled SEM, n = 6 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the SNK test.

Table 3.7 Free amino acids of plasma of hybrid striped bass at 6 hours postprandial

	60%FM	45%FM	30%FM	15%FM	3%FM	0%FM	P-value	Pooled SEM
Asp	32.1[b]	37.6[ab]	42.2[ab]	47.9[a]	44.4[a]	44.1[ab]	0.02	4.51
Glu	125	125	142	151	159	129	0.12	15.0
Asn	104[b]	127[ab]	144[a]	156[a]	153[a]	166[a]	0.00	14.3
Ser	183[c]	202[c]	237[bc]	249[bc]	322[a]	279[ab]	0.00	26.7
Gln	224[c]	245[b]	241[b]	268[ab]	276[ab]	266[ab]	0.00	23.1
His	436[a]	294[b]	322[b]	273[b]	202[c]	189[c]	0.00	24.4
Gly	456	522	520	513	555	595	0.11	46.6
Thr	202	224	230	236	248	211	0.31	21.1
Cit	48.7[a]	47.9[a]	40.3[ab]	33.9[bc]	31.1[bc]	25.8[c]	0.00	4.62
Arg	172	164	168	159	151	137	0.42	17.4
Tau	1630	1490	1530	1480	1290	1310	0.21	158
Ala	1240	1190	1250	1340	1420	1340	0.07	120
Tyr	145	165	174	166	185	167	0.21	15.1
Trp	35.8[c]	44.9[c]	44.5[bc]	54.9[ab]	61.5[a]	62.7[a]	0.00	4.72
Met	153	152	138	146	134	126	0.27	12.8
Val	520	536	564	507	475	445	0.23	49.9
Phe	184	204	176	208	191	187	0.73	21.4
Ile	268	273	300	283	299	293	0.84	30.1
Leu	461	438	417	432	459	415	0.85	45.2
Orn	39.6	41.0	42.4	38.1	39.3	40	0.94	4.25
Lys	301	292	266	289	276	262	0.63	26.1
Cys	14.4	14.2	15.3	15.6	16.4	15.1	0.85	1.8.1
Hyp	140	154	143	148	137	139	0.88	15.1
Pro	316	293	292	299	285	290	0.90	27.2
Asp+ Glu+ Gln+ Ala	1621[bc]	1597[c]	1675[b]	1807[a]	1899[a]	1779[ab]	0.00	128
EAA[1]	2733[a]	2627[ab]	2626 [ab]	2588 [ab]	2497 [ab]	2328[b]	0.01	233
Total	7017	6850	7052	7008	7001	6714	0.51	221

1: Essential amino acids: HIS, THR, ARG, TRP, MET, VAL, PHE, ILE, LEU, and LYS.
Values, expressed nmol/ml, are means and pooled SEM, n = 6 per treatment group.
a-c: Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the SNK test.

Discussion

The present study successfully demonstrated that the combination of high protein animal-origin and plant-origin ingredients could replace up to 75% of FM in the HSB diets without any adverse effects on growth performance of juvenile HSB, which was possibly due to their abundance of tranditionally nonessential AAs, especially the high levels of glutamine and glutamate in SPC.

Soy protein concentrate and PBM may be promising protein sources to replace FM in the diets of fish. SPC not only has a crude protein level similar to that of FM, but also has equal or greater AA levels than FM except for methionine and lysine (U.S. Soybean Export Councel 2008). SPC contains no or a very limited amount of anti-nutritional factors as well as indigestible and harmful carbohydrates (components of soybean meal), which have been almost completely extracted or removed during the production of SPC from soybeans (U.S. Soybean Export Councel 2008). Therefore, SPC is more palatable for fish than soybean meal. One study found that rainbow trout fed a diet with FM being completely replaced by SPC but with supplemental methionine exhibited a growth rate similar to that of fish fed an FM-based diet (Kaushik et al. 1995). Other studies demonstrated that SPC could replace 25-75% of FM in the diets for rainbow trout, Atlantic halibut, Atlantic salmon, and turbot without adverse effects on their growth performance (Médale et al. 1998; Mambrini et al. 1999a; Berge et al. 1999; Storebakken et al. 2000; Day and GonzAlez 2000). Notably, SPC contains the highest amount of glutamate and glutamine among the most common protein source ingredients (Li et al. 2011; USDA 2018). For example, the content of glutamate and glutamine in SPC is 37% greater than that in FM (Table 3.1) (USDA, 2018). Thus, the concentrations of glutamine and glutamate in plasma at both 5 and 18 h post feeding increased when the content of SPC in the FM-replaced diet for Atlantic cod and rainbow trout increased (Mambrini et al. 1999b; Hansen et al. 2007). Weight gain, feed efficiency, protein retention,

intestinal histological structures, and digestive enzyme activities were reported to be improved for Jian carp (Liu and Zhou 2006), hybrid sturgeon (Xu et al. 2011), gilthead seabream (Caballero-Solares et al. 2015), and red drum (Cheng et al. 2011). Consistent with these findings, we found that SPC was a promising alternative to FM in HSB diets.

As noted in Chapter 1, poultry byproducts provide taurine and creatine (as FM does) that are absent from SPC. Thus, the combination of both PBM and SPC may provide a complementary mixture of AAs in diets. However, the content of some EAAs in PBM and SPC is usually lower than that in FM. This view is consistent with the previous findings of FM replacement studies that the levels of some EAAs (lysine, methionine, threonine, and taurine) in FM-replaced diets were insufficient for maximal growth of fish due to the low levels of limiting AAs in FM alternatives, although the amounts of those AAs met the published requirement estimates (Fournier et al. 2003, 2004; Rawles et al. 2009). Therefore, the idea of formulating diets based on the "ideal" AA profile of FM in FM-replaced diets has been postulated (Mambrini and Kaushik 1995). A study showed that HSB that were fed a diet containing EAA levels similar to those in FM and NEAAs outperformed fish that were fed a diet formulated to meet the NRC-recommended EAA requirements (Twibell et al. 2003). Clearly, because NEAAs (including glutamate and glutamine) serve enormous functions in animals (including fish), these AAs must be considered when diets are formulated (Wu et al. 2013b; Hou et al. 2015).

Based on the result of this study, an HSB diet that replaces up to 75% of dietary FM with PBM and SPC and contains crystalline AA supplements appeared to be as effective as the diet containing FM as the sole protein source. In fact, HSB that were fed a diet containing a mixture of FM, PBM, and SPC as protein sources and supplemented with certain AAs (threonine, lysine, methionine, and taurine) could grow better than fish were fed a diet with FM as the only protein source. Lysine and methionine were the first limiting AA in PBM and

SPC, respectively (U.S. Soybean Export Councel 2008; Oliva-Teles et al. 2015). Of all EAAs listed in Table 3.1, the content of threonine in PMB and SPC was about 50% lower than that in FM. One study found that threonine was the first limiting AA after lysine and methionine in an FM-PBM based diet for HSB (Rawles et al. 2009). Although taurine is not a proteinogenic AA, a deficiency of taurine in diet could impair the growth performance of fish fed high levels of low-taurine ingredients (Salze and Davis 2015).

The growth performance of HSB in all groups was generally better than that reported from studies that used either PBM, soybean product (soybean meal, SPC, etc.) or both to replace FM in diets for HSB (Gallagher 1994; Brown et al. 1997; Rawles et al. 2006, 2009; Gaylord and Rawles 2007; Blaufuss and Trushenski 2012). The first possible reason is that 3-month-old HSB with a body weight of approximately 5.5 gram in the present study had a much faster growth rate than the fish in some other studies that were much larger and older (Trushenski and Gause 2013). The second reason could be that fish were fed four times a day at 7-8% of their body weight, compared with other studies where fish were fed once or twice daily at 4% or less of their body weight (Rawles et al. 2006, 2009). The growth rates and basal metabolic rates of fingerling fish were higher than those of older fish when provided adequate nutrients (Hopkins 1992). Fish that were fed three or four times a day showed the greatest growth and improved FCR than fish fed the same amount of feed once or twice daily (Wang et al. 1998). Therefore, the SGR, FCR, and PER of fish in all groups were much better than those in the previous studies. In addition, our feeding strategy allowed for a greater portion of dietary protein to be used for body growth and thus was better to demonstrate potential differences in the anabolic efficacy of test diets. However, in terms of practice in aquaculture industry, it may be only applicable for the fast growth stage of fingerling at very young age because of the time-consuming feeding frequency.

An interesting observation from the present study is that the growth performance of HSB that were fed diets with 45% FM or 30% FM was better than that of fish fed the 60% FM diet. This result contradicted most FM replacement studies, in which fish fed a 60% FM diet grew better than or equally to FM-replaced groups (Brown et al. 1997, Pine et al. 2008). Many of those FM replacement studies only considered the first limiting AA, such as methionine, but did not check other critical AAs that were much lower in alternative protein sources than those in FM (Gallagher 1994; Brown et al. 1997; Rawles et al. 2009; Trushenski and Gause 2013). Thus, those studies could underestimate the potential of FM alternatives to replace FM in fish diets. Therefore, in the present study, not only the first limiting AAs (lysine and methionine) of PBM and SPC, but also threonine and taurine, were supplemented to the FM-replaced diets based on the differences in the AA profile of PBM, SPC, and FM (Li et al. 2011). Of those four AAs, taurine is traditionally classified as a nonessential AA for fish. However, a number of studies demonstrated that a deficiency of taurine reduced growth performance in fish when high levels of FM were replaced by ingredients that lacked taurine, such as plant meals (Lunger et al. 2007; Chatzifotis et al. 2008). Another reason for a better growth performance of fish fed diets with 45% FM or 30% FM could be that a mixture of protein ingredients was included in our diets. It is also possible that FM provided an excess of an AA (e.g., methionine) that resulted in an imbalance among AAs in the diet. Several FM replacement studies demonstrated that including alternative protein ingredients in conventional FM-based diets could have superior performance in different fish species. For example, Rawles, et al (2009) found that the growth performance of HSB fed a diet containing 0% FM, 34.80% PBM, and 25.90% soybean meal was similar to that of fish fed the control diet containing 25% FM, 7.73% PBM, and 25.90% soybean meal. Another study even showed that juvenile barramundi fed diets containing different alternative protein ingredients had better growth performance than fish fed the control FM diet in terms of

weight gain, feed intake, and feed conversion (Glencross et al. 2011). Interestingly, the diet with about 50% FM replacement by a mixture of poultry offal meal and narrowleaf lupin kernel meal showed the best growth performance (Glencross et al. 2011). Those studies might be species-specific, but they demonstrated that the AA profile of a mixture of FM and other protein ingredients could be more "ideal" than that of FM as the sole source of dietary protein for some fish.

Fishmeal replacement studies have traditionally focused on EAA profiles, but the levels of NEAAs in fish diets were often omitted or considered unimportant for fish growth or health. Although the levels of glutamine + glutamate in the experimental diets did not differ substantially (5.97 % for 60 % FM; 6.13 % for 45% FM; 6.29 for 30% FM; 6.45 for 15 % FM; 6.59 for 3% FM; 6.61for 0% FM, as shown in Table 3.3). Our data on the concentrations of AAs in plasma at 6 h post feeding clearly showed an increasing trend for glutamine and asparagine as well as the sum of glutamine, glutamate, aspartate, and alanine in fish fed a diet with a mixture of PBM and SPC (Table 3.7). Interestingly, two studies also found a clear increase in the concentrations of glutamine or glutamate in the plasma of Atlantic cod and rainbow trout when they were fed diets with FM being replaced by either SPC or a mixture of SPC plus soybean meal plus wheat gluten. This indicated that a greater portion of glutamine, glutamine, alanine, or aspartate in SPC could enter the blood in HSB, or the extraintestinal tissues (e.g., skeletal muscle and liver) of HSB may synthesize more glutamine or glutamate from SPC- and poultry byproduct-derived AAs.

As indicated in Chapters 2, glutamate and glutamine play an important role in the nutrition and metabolism of juvenile HSB. Therefore, the higher content amount of glutamate, glutamine, alanine, and aspartate might contribute more ATP to fish because HSB tissues could preferentially use glutamine and glutamate as major energy sources (Jia et al. 2017). In addition, dietary supplementation with 0.5-2% glutamine could improve the growth

performance of Jian carp, red drum, and HSB (Cheng et al. 2011, 2012; Liu et al. 2015). Thus, the improved growth performance of HSB fed the 45 % FM and 30 % FM diets could be related to a higher concentration of glutamine in the plasma.

As reported by other investigators, a lower growth performance was found in HSB fed low FM diets. It could be caused by low palatability of diets with high levels of SPC and PBM, or by a lack of dietary taurine. Blaufuss and Trushenski (2012) found that SPC could affect the palatability of fish diets when it replaced only 33% FM in the diet, and thus the reduced growth rate of fish fed diets containing high levels of SPC appeared to be related only to the palatability of the feeds. A similar result was also observed in the present study: FCR, PER, and SGR became lower in the diets containing only 5% or 0% FM compared with other diets. Therefore, to solve the palatability problem of SPC when replacing FM in the diet, the combination of half PBM and half SPC plus the AA supplement to replace up to 75% FM in the diet appeared not to affect its palatability based on weight gain, SGR, and FCR data (Table 3.5). Because SPC used in the feeding trial contained very low anti-nutritional factors, it was unlikely that a decrease in the growth performance of HSB was caused by them when there was a high percentage of replacement by PBM and SPC. The fact that visceral fat index (VFI) decreased as dietary FM decreased probably indicated that FM could stimulate lipid synthesis to a greater extent than a mixture of PBM and SPC, possibly due to the different digestibility of the protein ingredients. This was also evidenced by the concentrations of histidine and total EAAs in plasma at 6 h post feeding, which decreased as FM decreased although all test diets were formulated to provide similar levels of EAAs (Table 3.2). The similar rates of muscle protein synthesis among all groups of fish suggested that the rates of muscle proteolysis may differ, resulting the different rates of protein gains in skeletal muscle (Figure 3.1).

Conclusion

The findings of this study indicated that FM in HSB diets could be successfully replaced by up to 75% with PBM and SPC along with AA supplementation without any adverse effect on growth performance. Interestingly, fish fed an AA-supplemented diet with 25-50% FM being replaced by PBM and SPC actually grew better than fish fed a diet with FM as the sole protein source. This improvement in growth performance could result from high concentrations of glutamine and other NEAAs (glutamate, alanine, and aspartate) in the plasma of HSB fed diets containing SPC. Glutamate and glutamine likely play an important role in successfully replacing FM in the diets of HSB.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The requirement of fish for dietary protein is generally higher than that of terrestrial animals although the whole body protein content amino acid composition of those animals is similar. Glutamine and glutamate, two traditionally nonessential amino acids, account for more than 10 % of amino acids in fishmeal that is an optimal dietary protein source for fish. So far it is still unclear on 1) Why such a high dietary protein requirement is needed for fish, especially carnivorous species; and 2) Whether using glutamine and glutamate-abundant soybean protein concentrate is a potential and promising way to replace fishmeal in hybrid striped bass (HSB). Two series of experiments were conducted to close this gap of knowledge.

Compared with leucine, glucose, and palmitate, higher *in vitro* oxidation rates of glutamine and glutamate for ATP production in the proximal intestine, liver, kidney and skeletal muscle of HSB indicated that dietary amino acids (primarily glutamate and glutamine) were their major metabolic fuels. Three amino acids used in the experiment, glutamate, glutamine, and leucine, together contributed to about 80% of ATP production in the fish tissues. The high rates of glutamate uptake and high activities of glutamate dehydrogenase, glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase in the HSB tissues provided a biochemical basis for explaining the importance of energy contribution from glutamate in the tissues. Lastly, in the fishmeal replacement study, two high protein ingredients (poultry byproduct meal and soy protein concentrate) along with crystalline amino acids were used to replace fishmeal in the HSB diet. Although digestibility decreased as dietary fishmeal decreased, the growth performance of fish fed a diet with up to 50 % fishmeal being replaced was better than that of the fish fed the conventional fishmeal-

based diet providing all dietary protein. Because the content of nutritionally essential amino acids and taurine was similar among the different diets, the enhanced growth of HSB fed the fishmeal-replaced diet might possibly result from the higher amount of dietary glutamine + glutamate because soy protein concentrate contained 37 % more glutamine plus glutamate than fishmeal.

The novel findings from this thesis will aid in the development of practical fishmeal replacement diets for carnivorous fish in the aquafeed industry and also provide a fundamental explanation of the high requirement of dietary protein for fish. The results not only bring up new research directions regarding the mechanisms for the beneficial effect of dietary glutamine and glutamate on HSB growth but also shed light on the importance of the diverse functions of the traditionally classified nonessential amino acids, such as glutamine and glutamate, for fish.

Although the present research helps us to understand the role of glutamate and glutamine as the major metabolic fuels in fish tissues, as well as the significance of dietary glutamine and glutamate for fish growth, some important questions remain. For example, 1) how glutamine and glutamate are oxidized in the live HSB *in vivo*, and 2) how glutamate and glutamine in fishmeal alternatives improve HSB growth. More studies are needed to comprehensively address these questions. First of all, *in-vivo* studies using radio-labeled diets would be more accurate to evaluate the contribution from different nutrients to ATP production because *in vitro* studies have their own limitations in extrapolation to the *in vivo* situation. Second, further research is needed to explain how dietary glutamine and glutamate improve the growth of fish. Therefore, with further investigations, there will be a better understanding of the functions of glutamine and glutamate in fish nutrition and metabolism.

REFERENCES

Abdel-Tawwab M, Ahmad MH, Khattab YAE, Shalaby AME (2010) Effect of dietary protein level, initial body weight, and their interaction on the growth, feed utilization, and physiological alterations of Nile tilapia, *Oreochromis niloticus* (L.). Aquaculture 298:267–274

Albrecht J, Norenberg MD (2006) Glutamine: A Trojan horse in ammonia neurotoxicity. Hepatology 44:788–794

Andersen S, Waagbø R, Espe M (2016) Functional amino acids in fish health and welfare. Front Biosci 8:143–169

Association of Official Analytical Chemists (1990). Official methods of analysis, 15th edn. Assoc Anal Chem, Washington, DC

Assaad HI, Zhou L, Carroll RJ, Wu G (2014) Rapid publication-ready MS-Word tables for one-way ANOVA. Springerplus 3:474

Baeverfjord G, Krogdahl A (1996) Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison with the intestines of fasted fish. J Fish Dis 19:375–387

Ballantyne JS (2001) Amino acid metabolism. Fish Physiol 20:77–107

Bartell SM, Batal AB (2007) The effect of supplemental glutamine on growth performance, development of the gastrointestinal tract, and humoral immune response of broilers. Poult Sci 86:1940–7

Berge GM, Grisdale-Helland B, Helland SJ (1999) Soy protein concentrate in diets for Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 178:139–148

Berk Z (1992) Technology of production of edible flours and protein products from soybeans. Food and Agriculture Organization of the United Nations (FAO), Rome

Bhaskar M (1994) Changes in the liver protein fractions of *Tilapia mossambica* (Peters) during acclimation to low and high pH media. Fish Res 19:179–186

Blair R (2018) Nutrition and feeding of organic poultry. Cabi Series. CABI, Wallingford

Blaufuss P, Trushenski J (2012) Exploring soy-derived alternatives to fish meal: Using soy protein concentrate and soy protein isolate in hybrid striped bass feeds. N Am J Aquac 74:8–19

Brander KM (2007) Global fish production and climate change. Proc Natl Acad Sci 104:19709–19714

Brosnan JT, Brosnan ME (2013) Glutamate: a truly functional amino acid. Amino Acids 45:413–418

Brown NF (2003) Expression, purification, and reconstitution of rat liver carnitine palmitoyltransferase I. In: Slinskey, BS (ed) Membrane Protein Protocols. Humana Press, New Jersey, pp 281–302

Brown PB, Twibell R, Jonker Y, Wilson KA (1997) Evaluation of Three Soybean Products in Diets Fed to Juvenile Hybrid Striped Bass *Morone saxatilis* × *M. chrysops*. J World Aquac Soc 28:215–223

Buentello JA, Gatlin DM (2001) Effects of elevated dietary arginine on resistance of channel catfish to exposure to *Edwardsiella ictaluri*. J Aquat Anim Health 13:194–201

Bush JA, Wu G, Suryawan A, et al (2002) Somatotropin-induced amino acid conservation in pigs involves differential regulation of liver and gut urea cycle enzyme activity. J Nutr 132:59–67

Caballero-Solares A, Viegas I, Salgado MC, et al (2015) Diets supplemented with glutamate or glutamine improve protein retention and modulate gene expression of key enzymes of hepatic metabolism in gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 444:79–87

Campbell JW, Aster PL, Vorhaben JE (1983) Mitochondrial ammoniagenesis in liver of the channel catfish *Ictalurus punctatus*. Am J Physiol Integr Comp Physiol 244:R709–R717

Chamberlin ME, Glemet HC, Ballantyne JS (1991) Glutamine metabolism in a holostean (*Amia calva*) and teleost fish (*Salvelinus namaycush*). Am J Physiol Integr Comp Physiol 260:R159–R166

Chan J (2016) Investigating hepatic glutamate dehydrogenase activity as a cause for the significant loss of amino acid utilization efficiency in growing rainbow trout (*Oncorhynchus mykiss*). Thesis, The University of Guelph

Chatzifotis S, Polemitou I, Divanach P, Antonopoulou E (2008) Effect of dietary taurine supplementation on growth performance and bile salt activated lipase activity of common dentex, *Dentex dentex*, fed a fish meal/soy protein concentrate-based diet. Aquaculture 275:201–208

Cheng Z, Buentello A, Gatlin DM (2011) Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*. Aquaculture 319:247–252

Cheng Z, Gatlin DM, Buentello A (2012) Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops*×*M. saxatilis*). Aquaculture 362–363:39–43

Christiansen DC, Klungsøyr L (1987) Metabolic utilization of nutrients and the effects of insulin in fish. Comp Biochem Physiol B 88:701–711

Costas B, Conceição LEC, Dias J, et al (2011) Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858). Fish Shellfish Immunol 31: 838–847

Cowey CB, Walton MJ (1988) Studies on the uptake of (^{14}C) amino acids derived from both dietary (^{14}C) protein and dietary (^{14}C) amino acids by rainbow trout, *Salmo gairdneri*

Richardson. J Fish Biol 33:293–305

Dai Z, Wu Z, Jia S, Wu G (2014a) Analysis of amino acid composition in proteins of animal tissues and foods as pre-column o-phthaldialdehyde derivatives by HPLC with fluorescence detection. J Chromatogr B 964:116–127

Dai Z, Wu Z, Wang J, et al (2014b) Analysis of polyamines in biological samples by HPLC involving pre-column derivatization with o-phthalaldehyde and N-acetyl-L-cysteine. Amino Acids 46:1557–1564

Davies, S.J., Williamson, J., Robinson, M. & Bateson, R.I. (1989) Practical inclusion levels of common animal by-products in complete diets for tilapia *Oreochromis mossambicus*, Peters. In: The Current Status of Fish Nutrition in Aquaculture Proceedings of the Third International Symposium on Feeding and Nutrition in Fish, Toba, Japan, pp. 325–332

Dawson AG, Hird FJR, Morton DJ (1967) Oxidation of leucine by rat liver and kidney. Arch Biochem Biophys 122:426–433

Day OJ, GonzAlez HGP (2000) Soybean protein concentrate as a protein source for turbot *Scophthalmus maximus* L. Aquac Nutr 6:221–228

Ebeling JM, Timmons MB (2012) Recirculating Aquaculture Systems. In: Tidwell, JH (ed) Aquaculture Production Systems. Wiley, Blackwell, pp. 245–277

Enes P, Panserat S, Kaushik S, Oliva-Teles A (2006) Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (*Dicentrarchus labrax*) juveniles. Comp Biochem Physiol Part A Mol Integr Physiol 143:89–96

Food and Agriculture Organization (2018) The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome

Fournier V, Gouillou-Coustans MF, Métailler R, et al (2003) Excess dietary arginine affects urea excretion but does not improve N utilisation in rainbow trout *Oncorhynchus mykiss*

and turbot *Psetta maxima*. Aquaculture 217: 559–576

Fournier V, Huelvan C, Desbruyeres E (2004) Incorporation of a mixture of plant feedstuffs as substitute for fish meal in diets of juvenile turbot (*Psetta maxima*). Aquaculture 236: 451–465

French CJ, MOMMSEN TP, HOCHACHKA PW (1981) Amino Acid Utilisation in Isolated Hepatocytes from Rainbow Trout. Eur J Biochem 113:311–317

Fynn-Aikins K, Hughes SG, Vandenberg GW (1995) Protein retention and liver aminotransferase activities in Atlantic salmon fed diets containing different energy sources. Comp Biochem Physiol Part A Physiol 111:163–170

Gallagher ML (1994) The use of soybean meal as a replacement for fish meal in diets for hybrid striped bass (*Morone saxatilis*×*M. chrysops*). Aquaculture 126:119–127

Gatlin DM, Barrows FT, Brown P, et al (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac Res 38:551–579

Gaylord TG, Barrows FT, Overturf K, et al (2010) An overview of progress toward developing an all plant-based diet for rainbow trout. Bull Fish Res Agency 31:9–14

Gaylord TG, Rawles SD (2007) The modification of poultry by-product meal for use in hybrid striped bass *Morone chrysops*×*M. saxatilis* Diets. J World Aquac Soc 36:363–374

Glencross B, Rutherford N, Jones B (2011) Evaluating options for fishmeal replacement in diets for juvenile barramundi (*Lates calcarifer*). Aquac Nutr 17:e722–e732

Glencross BD, Booth M, Allan GL (2007) A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. Aquac Nutr 13:17–34

Griffin ME, Wilson KA, White MR, Brown PB (1994) Dietary choline requirement of juvenile hybrid striped bass. J Nutr 124:1685–1689

Hansen AC, Rosenlund G, Karlsen Ø, et al (2007) Total replacement of fish meal with plant

proteins in diets for Atlantic cod (*Gadus morhua* L.) I - Effects on growth and protein retention. Aquaculture 272:599–611

Hardy R (2010) Utilization of plant proteins in fish diets: Effects of global demand and supplies of fishmeal. Aquac. Res. 41: 770–776

Harrell R. (2016) FAO Fisheries and Aquaculture - Cultured aquatic species information Programme - *Morone* hybrid. In: FAO Fish. Aquac. Dep. http://www.fao.org/fishery/culturedspecies/Morone_hybrid/en. Accessed 8 Mar 2019

Haynes TE, Li P, Li X, et al (2009) L-Glutamine or L-alanyl-L-glutamine prevents oxidant- or endotoxin-induced death of neonatal enterocytes. Amino Acids 37: 131–142

Haÿs SP, Ordonez JM, Burrin DG, Sunehag AL (2007) Dietary glutamate is almost entirely removed in its first pass through the splanchnic bed in premature infants. Pediatr Res 62:353–356

Hemre GI, Mommsen TP, Krogdahl Å (2002) Carbohydrates in fish nutrition: Effects on growth, glucose metabolism and hepatic enzymes. Aquac Nutr 8:175–194

Hill JC, Alam MS, Watanabe WO, et al (2019) Replacement of menhaden fish meal by poultry by-product meal in the diet of juvenile red porgy. N Am J Aquac 81:81–93

Hopkins KD (1992) Reporting fish growth: a review of the basics. J World Aquac Soc 23:173–179

Hou Y, Hu S, Jia S, et al (2016) Whole-body synthesis of l-homoarginine in pigs and rats supplemented with l-arginine. Amino Acids 48:993–1001

Hou Y, Wu G (2018) l-Glutamate nutrition and metabolism in swine. Amino Acids 50:1497–1510

Hou Y, Yin Y, Wu G (2015) Dietary essentiality of "nutritionally non-essential amino acids" for animals and humans. Exp Biol Med 240:997–1007

Houlihan D, Boujard T, Malcom J (2001) Food Intake in Fish. Blackwell Science, Oxford.

Hughes SG, Rumsey GL, Nesheim MC (1983) Branched-chain amino acid aminotransferase activity in the tissues of lake trout. Comp Biochem Physiol 76B:429–431

Ip YK, Chew SF (2010) Ammonia production, excretion, toxicity, and defense in fish: A review. Front Physiol 1:1–20

Jia S, Li X, Zheng S, Wu G (2017) Amino acids are major energy substrates for tissues of hybrid striped bass and zebrafish. Amino Acids 49:2053–2063

Jobgen WS, Fried SK, Fu WJ, et al (2006) Regulatory role for the arginine–nitric oxide pathway in metabolism of energy substrates. J Nutr Biochem 17:571–588

Jürss K, Bastrop R (1995) Amino acid metabolism in fish. In: Hochachka PW, Mommsen TP (eds) Biochemistry and Molecular Biology of Fishes. pp 159–189

Kats LJ, Laurin JL, Tokach MD, et al (1992) Comparison of spray-dried blood meal and fish by-products in the phase II starter pig diet. Kansas Agric Exp Stn Res Reports 37–40, Manhattan

Kaushik SJ, Cravedi JP, Lalles JP, et al (1995) Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 133:257–274

Kaushik SJ, Seiliez I (2010) Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. Aquac Res 41:322–332

Kong X, Tan B, Yin Y, et al (2012) l-Arginine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells. J Nutr Biochem 23:1178–1183

Krogdahl Å, Penn M, Thorsen J, et al (2010) Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquac Res 41:333–344

Laale HW (1977) The biology and use of zebrafish, *Brachydanio rerio* in fisheries research..

A literature review. J Fish Biol 10:121–173

Latshaw JD, Bishop BL (2001) Estimating body weight and body composition of chickens by using noninvasive measurements. Poult Sci 80:868–73

Lee DJW, McNab JM, Shannon DWF, Blair R (1972) Enzyme studies with the livers of chicks fed semi-synthetic diets containing crystalline amino acids and diammonium citrate. Br Poult Sci 13:229–235

Lei J, Feng D, Zhang Y, et al (2012) Regulation of leucine catabolism by metabolic fuels in mammary epithelial cells. Amino Acids 43:2179–2189

Lei J, Feng D, Zhang Y, et al (2013) Hormonal regulation of leucine catabolism in mammary epithelial cells. Amino Acids 45:531–541

Lenis YY, Wang X, Tang W, et al (2016) Effects of agmatine on secretion of interferon tau and catecholamines and expression of genes related to production of polyamines by ovine trophectoderm cells. Amino Acids 48: 2389–2399

Li H, Meininger CJ, Bazer FW, Wu G (2016) Intracellular sources of ornithine for polyamine synthesis in endothelial cells. Amino Acids 48:2401–2410

Li P, Knabe DA, Kim SW, et al (2009a) Lactating porcine mammary tissue catabolizes branched-chain amino acids for glutamine and aspartate synthesis. J Nutr 139:1502–1509

Li P, Mai K, Trushenski J, Wu G (2009b) New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids 37:43–53

Li X, Bazer FW, Johnson GA, et al (2010) Dietary supplementation with 0.8% L-arginine between days 0 and 25 of gestation reduces litter size in gilts. J Nutr 140: 1111–1116

Li X, Rezaei R, Li P, et al (2011) Composition of amino acids in feed ingredients for animal diets. Amino Acids 40:1159–1168

Liu J, Mai K, Xu W, et al (2015) Effects of dietary glutamine on survival, growth

performance, activities of digestive enzyme, antioxidant status and hypoxia stress resistance of half-smooth tongue sole (*Cynoglossus semilaevis* Günther) post larvae. Aquaculture 446:48–56

Liu XD, Wu X, Yin YL, et al (2012) Effects of dietary l-arginine or N-carbamylglutamate supplementation during late gestation of sows on the miR-15b/16, miR-221/222, VEGFA and eNOS expression in umbilical vein. Amino Acids 42:2111–2119

Lobley GE, Milne V, Lovie JM, et al (1980) Whole body and tissue protein synthesis in cattle. Br J Nutr 43:491–502

Lougheed M, Nelson B (2001) Hybrid striped bass production, markets and marketing. Michigan State University, East Lansing

Lunger AN, McLean E, Gaylord TG, et al (2007) Taurine supplementation to alternative dietary proteins used in fish meal replacement enhances growth of juvenile cobia (*Rachycentron canadum*). Aquaculture 271:401–410

MacLennan PA, Brown RA, Rennie MJ (1987) A positive relationship between protein synthetic rate and intracellular glutamine concentration in perfused rat skeletal muscle. FEBS Lett 215:187–191

Mambrini M, Kaushik SJ (1995) Indispensable amino acid requirements of fish: correspondence between quantitative data and amino acid profiles of tissue proteins. J Appl Ichthyol 11:240–247

Mambrini M, Roem AJ, Carvèdi JP, et al (1999) Effects of replacing fish meal with soy protein concentrate and of DL-methionine supplementation in high-energy, extruded diets on the growth and nutrient utilization of rainbow trout, *Oncorhynchus mykiss*. J Anim Sci 77:2990–2999

Mateo RD, Wu G, Moon HK, et al (2008) Effects of dietary arginine supplementation during gestation and lactation on the performance of lactating primiparous sows and nursing

piglets1. J Anim Sci 86:827–835

Médale F, Boujard T, Vallée F, et al (1998) Voluntary feed intake, nitrogen and phosphorus losses in rainbow trout (*Oncorhynchus mykiss*) fed increasing dietary levels of soy protein concentrate. Aquatic Living Resources. 11: 239–246

Merino G, Barange M, Blanchard JL, et al (2012) Can marine fisheries and aquaculture meet fish demand from a growing human population in a changing climate? Glob Environ Chang 22:795–806

National Research Council (2000) Nutrient Requirements of Beef Cattle. National Academies Press, Washington, D.C.

National Research Council (2012) Nutrient Requirements of Swine. National Academies Press, Washington, D.C.

National Research Council (2011) Nutrient requirements of fish and shrimp. National Academies Press, Washington, D.C.

Nengas I, Alexis MN, Davies SJ (1999) High inclusion levels of poultry meals and related byproducts in diets for gilthead seabream *Sparus aurata* L. Aquaculture 179:13–23

Oliva-Teles A, Enes P, Peres H (2015) Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. In: Davis D (ed) Feed and Feeding Practices in Aquaculture. Elsevier, Kidlington, pp 203–233

Olsen RL, Hasan MR (2012) A limited supply of fishmeal: Impact on future increases in global aquaculture production. Trends Food Sci Technol 27:120–128

Pine HJ, Daniels WH, Davis DA, et al (2008) Replacement of fish meal with poultry by-product meal as a protein source in pond-raised sunshine bass, *Morone chrysops* ♀ × *M. saxatlis* ♂, Diets. J World Aquac Soc 39:586–597

Polakof S, Álvarez R, Soengas JL (2010) Gut glucose metabolism in rainbow trout: implications in glucose homeostasis and glucosensing capacity. Am J Physiol Integr

Comp Physiol 299:R19–R32

Qiyou X, Qing Z, Hong X, et al (2011) Dietary glutamine supplementation improves growth performance and intestinal digestion/absorption ability in young hybrid sturgeon (*Acipenser schrenckii* ♀ × *Huso dauricus*♂). J Appl Ichthyol 27:721–726

Quagrainie K (2015) Profitability of hybrid striped bass cage aquaculture in the midwest. Purdue Univeristy Extension Publication # EC-798-W / Illinois-Indiana Sea Grant Publication #15-004. https://www.edustore.purdue.edu/item.asp?Item_Number=EC-798-W. Accessed 25 October 2019

Rawles SD, Gaylord TG, McEntire ME, Freeman DW (2009) Evaluation of poultry by-product meal in commercial diets for hybrid striped bass, *Morone chrysops* ♀ × *Morone saxatilis* ♂, in Pond Production. J World Aquac Soc 40:141–156

Rawles SD, Riche M, Gaylord TG, et al (2006) Evaluation of poultry by-product meal in commercial diets for hybrid striped bass (*Morone chrysops* ♀×*M. saxatilis* ♂) in recirculated tank production. Aquaculture 259:377–389

Refstie S, Storebakken T, Baeverfjord G, Roem AJ (2001) Long-term protein and lipid growth of Atlantic salmon (*Salmo salar*) fed diets with partial replacement of fish meal by soy protein products at medium or high lipid level. Aquaculture 193:91–106

Rønnestad I, Fyhn HJ (1993) Metabolic aspects of free amino acids in developing marine fish eggs and larvae. Rev Fish Sci 1:239–259

Rønnestad I, Thorsen A, Finn RN (1999) Fish larval nutrition: A review of recent advances in the roles of amino acids. Aquaculture 177:201–216

Rossignol O, Dodson JJ, Guderley H (2011) Relationship between metabolism, sex and reproductive tactics in young Atlantic salmon (*Salmo salar* L.). Comp Biochem Physiol A 159:82–91

Salze GP, Davis DA (2015) Taurine: a critical nutrient for future fish feeds. Aquaculture

437:215–229

Self JT, Spencer TE, Johnson GA, et al (2004) Glutamine synthesis in the developing porcine placenta1. Biol Reprod 70:1444–1451

Sidell BD, Crockett EL, Driedzic WR (1995) Antarctic fish tissues preferentially catabolize monoenoic fatty acids. J Exp Zool 271:73–81

Smits CHM, Moughan PJ, Smith WC (1988) Chemical whole-body composition of the 20 kg liveweight growing pig. New Zeal J Agric Res 31:155–157

Stickney RR, Hardy RW, Koch K et al (1996) The effects of substituting selected oilseed protein concentrates for fish meal in rainbow trout *Oncorhynchus mykiss* diets. J World Aquac Soc 27:57–63

Stone DA (2003) Dietary carbohydrate utilization by fish. Rev Fish Sci 11:337–369

Stoner GR, Allee GL, Nelssen JL, et al (1990) Effect of select menhaden fish meal in starter diets for pigs. J Anim Sci 68:2729–2735

Storebakken, Shearer, Roem (2000) Growth, uptake and retention of nitrogen and phosphorus, and absorption of other minerals in Atlantic salmon Salmo salar fed diets with fish meal and soy-protein concentrate as the main sources of protein. Aquac Nutr 6:103–108

Tacon AG, Hasan MR, Metian M (2011) Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects. FAO Fisheries and Aquaculture technical paper. No.564. Rome

Teigland M, Klungsøyr L (1983) Accumulation of α-ketoisocarproate from leucine in homogenates of tissues from rainbow trout (*Salmo gairdnerii*) and rat. An improved method for determination of branched chain keto acids. Comp Biochem Physiol B 75:703–705

Tng YYM, Wee NLJ, Ip YK, Chew SF (2008) Postprandial nitrogen metabolism and

excretion in juvenile marble goby, *Oxyeleotris marmorata* (Bleeker, 1852). Aquaculture 284:260–267

Troell M, Naylor RL, Metian M, et al (2014) Does aquaculture add resilience to the global food system? Proc Natl Acad Sci 111:13257–13263

Trushenski J, Gause B (2013) Comparative value of fish meal alternatives as protein sources in feeds for hybrid striped bass. N Am J Aquac 75:329–341

Twibell RG, Griffin ME, Martin B, et al (2003) Predicting dietary essential amino acid requirements for hybrid striped bass. Aquac Nutr 9:373–381

U.S. Department of Agriculture (2019) FoodData Central, USDA, Agricultural Research Service. https://fdc.nal.usda.gov/ . Accessed 25 October 2019

U.S. Soybean Export Council (2008) Soy protein concentrate for aquaculture feeds. Technical Bulletin US soybean Export Council, St. Louis.

van den Thillart G (1986) Energy metabolism of swimming trout (*Salmo gairdneri*) - Oxidation rates of palmitate, glucose, lactate, alanine, leucine and glutamate. J Comp Physiol B 156:511–520

Van Waarde A (1983) Aerobic and anaerobic ammonia production by fish. Comp Biochem Physiol B 74:675–684

Van Waarde A, Kesbeke F (1982) Nitrogen metabolism in goldfish, *Carassius auratus* (L.) activities of amidases and amide synthetases in goldfish tissues. Comp Biochem Physiol B 71:599–603

Wang N, Hayward RS, Noltie DB (1998) Effect of feeding frequency on food consumption, growth, size variation, and feeding pattern of age-0 hybrid sunfish. Aquaculture 165:261–267

Watford M, Wu G (2005) Glutamine metabolism in uricotelic species: variation in skeletal muscle glutamine synthetase, glutaminase, glutamine levels and rates of protein

synthesis. Comp Biochem Physiol B 140:607–614

Weber JM, Haman F (1996) Pathways for metabolic fuels and oxygen in high performance fish. Comp Biochem Physiol A 113:33–38

Wijayasinghe MS, Milligan LP, Thompson JR (1983) In vitro degradation of leucine in muscle, adipose tissue, liver, and kidney of fed and starved sheep. Biosci Rep 3:1133–1140

Wilson RP (2003) Amino acids and proteins. In: Halver JE, Hardy RW (eds) Fish Nutrition. Academic, San Diego, pp 143–179

Wu G (1997) Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. Am J Physiol 272:G1382–G1390

Wu G (1998) Intestinal mucosal amino acid catabolism. J Nutr 128:1249–1252

Wu G (2010) Functional amino acids in growth, reproduction, and health. Adv Nutr 1:31–37

Wu G (2013a) Amino Acids: biochemistry and nutrition. CRC, Boca Raton

Wu G (2013b) Functional amino acids in nutrition and health. Amino Acids 45:407–411

Wu G (2014) Dietary requirements of synthesizable amino acids by animals: a paradigm shift in protein nutrition. J Anim Sci Biotechnol 5:34

Wu G (2017) Principles of animal nutrition. CRC, Boca Raton

Wu G, Bazer FW, Burghardt RC, et al (2011) Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids 40:1053–1063

Wu G, Bazer FW, Dai Z, et al (2014) Amino acid nutrition in animals: protein synthesis and beyond. Annu Rev Anim Biosci 2:387–417

Wu G, Bazer FW, Johnson GA, Hou Y (2018) BOARD-INVITED REVIEW: Arginine nutrition and metabolism in growing, gestating, and lactating swine. J Anim Sci 96:5035–5051

Wu G, Bazer FW, Satterfield MC, et al (2013a) Impacts of arginine nutrition on embryonic

and fetal development in mammals. Amino Acids 4:241–256

Wu G, Haynes TE, Li H, Meininger CJ (2000) Glutamine metabolism in endothelial cells: ornithine synthesis from glutamine via pyrroline-5-carboxylate synthase. Comp Biochem Physiol A 126:115–123

Wu G, Knabe DA, Flynn NE (1994) Synthesis of citrulline from glutamine in pig enterocytes. Biochem J 299:115–121

Wu G, Meier SA, Knabe DA (1996) Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. J Nutr 126:2578–84

Wu G, Thompson JR (1987) Ketone bodies inhibit leucine degradation in chick skeletal muscle. Int J Biochem 19:937–943

Wu G, Thompson JR (1990) The effect of ketone bodies on protein turnover in isolated skeletal muscle from the fed and fasted chick. Int J Biochem 22:263–268

Wu G, Thompson JR, Baracos VE (1991) Glutamine metabolism in skeletal muscles from the broiler chick (*Gallus domesticus*) and the laboratory rat (*Rattus norvegicus*). Biochem J 274:769–774

Wu G, Wu Z, Dai Z, et al (2013b) Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. Amino Acids 44:1107–1113

Yan L, Qiu-Zhou X (2006) Dietary glutamine supplementation improves structure and function of intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquaculture 256:389–394

Yoshida C, Maekawa M, Bannai M, Yamamoto T (2016) Glutamate promotes nucleotide synthesis in the gut and improves availability of soybean meal feed in rainbow trout. Springerplus 5:1021

Zielke HR, Zielke CL, Ozand PT (1984) Glutamine: a major energy source for cultured mammalian cells. Fed Proc 43:121–125

APPENDIX A

Ammonia Assay (UV Spectrophotometric Method):

A. Chemicals:

1. TEA buffer (0.5 M), ADP (1.82 mM) and α-ketoglutarate (35 mM): Dissolve 4.65 g TEA-HCl, 47.5 mg ADP, sodium salt and 335 mg α-ketoglutaric acid in 40 ml H_2O, adjust to pH 8.0 with 10 M NaOH (~2.0 ml), and make up to 50 ml with H_2O.
2. ß-NADH (3 mM): Dissolve 15 mg ß-NADH, disodium salt, and 30 mg $NaHCO_3$ in 6 ml H_2O. Use a brown bottle to protect the solution against light.
3. Glutamate dehydrogenase (1200 KU/L): Use stock solution of the enzyme, undiluted.
4. NH_4Cl stock solution (200 nmol/ml).

B. Assay Procedures.

1. Reaction mixture for each 340-nm cuvette:

   1.0 ml TEA buffer/ADP/α-ketoglutarate

   50 µl ß-NADH

   0.5 ml Standard/Sample (pH 7.0)

2. Mix thoroughly. After 5 min, read absorbance A_1 at 340 nm.
3. Add 10 µl of enzyme solution into each cuvette.
4. Mix thoroughly. After 15 min, read absorbance A_2 at 340 nm.

(Reaction is completed by 15 min at room temperature as A_2 is constantwhen measured 5 min later)

L-Lactate Assay Using Spectrophotometric Method

A. Chemicals:
   1. Glycine buffer: Dissolve 3.8 g glycine and 0.2 g EDTA-Na in 80 ml H_2O (6 mM EDTA). Add 4.25 ml Hydrazine-monohydrate (99.8%). Adjust to pH 9.5 with 0.8 ml of 10 M NaOH. Make up to 100 ml with H_2O.
   2. 7.5 mM ß-NAD^+: Dissolve 50 mg ß-NAD^+ in 10 ml H_2O.
   3. L-Lactate dehydrogenase (5 mg/ml): Dilute the enzyme x10 with glycine buffer.
   4. Glycine-NAD solution: Mix 15 ml glycine buffer with 1 ml of 7.5 mM ß-NAD^+.
   5. L-Lactate standard

B. Assay Procedures.
   1. To each well, add the following:

      100 µl of Glycine-NAD solution

      10 µl L-lactate standard or Sample

   2. Mix well. After 5 min, read absorbance A_1 at 340 nm.
   3. Add 5 µl of L-lactate dehydrogenase into each well.
   4. Mix thoroughly. After 10 min, read absorbance A_2 at 340 nm.

(The reaction is completed by 10 min at room temperature)

Determination of Glutamate Dehydrogenase (GDH)Activity

A. Chemicals
   1. 80 mM Sodium phosphate buffer (pH 7.6):
      80 mM Na_2HPO_4: Dissolve 9.1 g Na_2HPO_4 (anhydrous) in 800 ml H_2O.
      80 mM NaH_2PO_4: Dissolve 2.21 g $NaH_2PO_4 \cdot H_2O$ in 200 ml H_2O.
      80 mM Sodium Phosphate buffer (pH 7.6): Mix the two solutions until pH 7.6.
   2. 4.8 mM ß-NADH/48 mM ADP: Dissolve 8.2 mg ß-NADH (disodium salt) and 52 mg ADP (disodium) in 2 ml of 80 mM sodium phosphate buffer (pH 7.6).
   3. 330 mM NH_4Cl: Dissolve 353 mg NH_4Cl in 20 ml of 80 mM sodium phosphate buffer (pH 7.6).
   4. Lactate dehydrogenase: Use stock solution undiluted.
   5. 210 mM α-Ketoglutarate: Dissolve 143 mg α-ketoglutarate (disodium salt) in 3 ml of 80 mM sodium phosphate buffer (pH 7.6).

B. Assay procedures:
   1. Add the following solution into a tube, and mix gently:

      1.7 ml 80 Mm Sodium Phosphate buffer (Ph 7.6)
      1.0 ml 330 mM NH4Cl
      0.1 ml 4.8 Mm NADH/48 Mm ADP solution
      0.1 ml standard or sample
      5 µl Lactate dehydrogenase
   2. Add 0.1 ml of 210 mM α-ketoglutarate to initiate the reaction. Measure absorbance at 340 nm every 1 min for 5 min, at 25 °C. For blanks, use 0.1 ml of 80 mM sodium phosphate buffer (pH 7.6) to replace 0.1 ml of 210 mM α-ketoglutarate.

Determination of Glutamate-Pyruvate Transaminase (GPT) Activity

A. Chemicals
   6. 80 mM Sodium phosphate buffer:
      80 mM Na_2HPO_4: Dissolve 9.1 g Na_2HPO_4 (anhydrous) in 800 ml H_2O.
      80 mM NaH_2PO_4: Dissolve 2.21 g $NaH_2PO_4 \cdot H_2O$ in 200 ml H_2O.
      80 mM Sodium Phosphate buffer (pH 7.6): Mix the two solutions until pH 7.6.
   7. 2.4 mM NADH: Dissolve 35 mg NADH (disodium salt) in 20 ml of 80 mM sodium phosphate buffer (pH 7.6).
   8. 210 mM α-Ketoglutarate: Dissolve 238 mg α-ketoglutarate (disodium salt) in 5 ml of 80 mM sodium phosphate buffer (pH 7.6).
   9. 92.3 mM Alanine: Dissolve in 822.4 mg L-alanine in 100 ml of 80 mM sodium phosphate buffer (pH 7.6).
   10. L-Lactate dehydrogenase: Use stock solution undiluted.

B. Assay Procedures:
   1. Add the following solution into a tube, and mix gently:
      2.6 ml 92 mM Aspartate
      0.2 ml 2.4 mM NADH
      5 µl Lactate dehydrogenase
      0.1 ml standard or sample
   2. Add 0.1 ml of 210 mM α-ketoglutarate to initiate the reaction. Measure absorbance at 340 nm every 1 min for 5 min, at 25 °C. For blanks, use 2.6 ml of 80 mM sodium phosphate buffer (pH 7.6) to replace 2.6 ml of 92.3 mM alanine.

Determination of Glutamate-Oxaloacetate Transaminase (GOT) Activity

A. Chemicals
    3. 80 mM Sodium phosphate buffer:
       80 mM Na_2HPO_4: Dissolve 9.1 g Na_2HPO_4 (anhydrous) in 800 ml H_2O.
       80 mM NaH_2PO_4: Dissolve 2.21 g $NaH_2PO_4 \cdot H_2O$ in 200 ml H_2O.
       80 mM Sodium Phosphate buffer (pH 7.6): Mix the two solutions until pH 7.6.
    4. 2.4 mM NADH: Dissolve 35 mg NADH (disodium salt) in 20 ml of 80 mM sodium phosphate buffer (pH 7.6).
    5. 210 mM α-Ketoglutarate: Dissolve 238 mg α-ketoglutarate (disodium salt) in 5 ml of 80 mM sodium phosphate buffer (pH 7.6).
    6. 38 mM Aspartate: Dissolve in 506 mg aspartic acid in 100 ml of 80 mM sodium phosphate buffer (pH 7.6).
    7. Malate dehydrogenase: Use stock solution undiluted.
B. Assay procedures:
    3. Add the following solution into a tube, and mix gently:
       2.6 ml 38 mM Aspartate
       0.2 ml 2.4 mM NADH
       5 µl Malate dehydrogenase
       0.2 ml standard or sample
    4. Add 0.1 ml of 210 mM α-ketoglutarate to initiate the reaction. Measure absorbance at 340 nm every 1 min for 5 min, at 25 °C. For blanks, use 2.6 ml of 80 mM sodium phosphate buffer (pH 7.6) to replace 2.6 ml of 38 mM aspartate.

Determination of Kidney-Type Phosphate-Dependent Glutaminase (K-GLS) Activity

A. Chemicals:
   1. 300 mM Potassium Phosphate Buffer (pH 8.2):
      a. 300 mM K_2HPO_4 : Dissolve 10.45 g K_2HPO_4 in 200 ml deionized H_2O.
      b. 300 mM KH_2PO_4 : Dissolve 8.17 g KH_2PO_4 in 200 ml deionized H_2O.
      c. 300 mM Pottassium Phosphate Buffer (pH 8.2): Mix 200 ml of 300 mM KH_2PO_4 with 300 mM K_2HPO_4 until the solution has pH 8.2.
   2. 40 mM Glutamine: Dissolve 29.3 mg glutamine in 5 ml of 300 mM potassium phosphate buffer (pH 8.2).

B. Assay Procedure:
   1. To each tube, add the following:
      250 µl of 40 mM glutamine
      200 µl H_2O
   2. Add 50 µl of sample to initiate the reaction.
   3. Incubate the assay mixture at 26 °C for 15 min.
   4. Terminate the reaction with 100 µl of 1.5 M $HClO_4$. After 2 min, neutralize the solution with 50 µl of 2 M K_2CO_3. Analyze glutamate using our HPLC method.

   Blanks: Blank tubes contain 250 µl 40 mM glutamine and 200 µl H_2O. Add 100 µl of 1.5 M $HClO_4$ before addition of samples.

Determination of Liver-Type Phosphate-Dependent Glutaminase (L-GLS) Activity

A. Chemicals:
   1. 300 mM Potassium Phosphate Buffer (pH 8.2):
      a. 300 mM K_2HPO_4 : Dissolve 10.45 g K_2HPO_4 in 200 ml deionized H_2O.
      b. 300 mM KH_2PO_4 : Dissolve 8.17 g KH_2PO_4 in 200 ml deionized H_2O.
      c. 300 mM Pottassium Phosphate Buffer (pH 8.0): Mix 200 ml of 300 mM KH_2PO_4 with 300 mM K_2HPO_4 until the solution has pH 8.0.
   2. 5 mM Hepes (pH 7.4): Dissolve 596 mg Hepes in 480 ml H_2O. Adjust to pH 7.4 with 10 M NaOH. Make up to a final volume of 500 ml with H_2O.
   3. 120 mM Tris-HCl buffer (pH 8.0): Dissolve 1.89 g Trizma-HCl in ~90 ml H_2O. Adjust pH to 8.0 with 1 M NaOH, and make up to a final volume of 100 ml with H_2O.
   4. 200 mM Glutamine: Dissolve 147 mg glutamine in 5 ml of H_2O.
   5. 24 mM NH_4Cl: Dissolve 64.2 mg NH_4Cl in 50 ml H_2O.

B. Assay Procedure:
   1. To each tube, add the following:
      100 µl of 300 mM potassium phosphate buffer (pH 8.0)
      100 µl of 120 mM Tris-HCl buffer (pH 8.0)
      300 µl of 200 mM glutamine
      50 µl of 24 mM NH_4Cl
   2. Add 50 µl of samples to initiate the reaction.
   3. Incubate the assay mixture at 26 °C for 20 min.
   4. Terminate the reaction with 100 µl

   Blanks: Blank tubes contain all assay components, but and 100 µl of 1.5 M $HClO_4$ before addition of samples.

Determination of Branched-Chain Amino Acid Transaminase (BCAAT) Activity in Tissue

A. Chemicals:
   1. 50 mM Hepes/KOH (pH 7.5): Dissolve 6 g Hepes (free acid) in 450 ml DD-H_2O. Adjust pH to 7.5 with 10 M KOH, and make up to a final volume of 500 ml with DD-H_2O.
   2. 50 mM Tris/HCl (pH 8.6): Dissolve 4 g Trizma-HCl in 450 ml of DD-H_2O. Adjust pH to 8.6 with 1 M NaOH, and make up to a final volume of 500 ml with H_2O.
   3. 50 mM α-Ketoglutarate: Dissolve 45 mg α-ketoglutarate in 4 ml of 50 mM Tris/HCl (pH 8.6).
   4. 1.6 mM Pyridoxal phosphate: Dissolve 4 mg pyridoxal phosphate (98%) in 10 ml of 50 mM Tris/HCl buffer (pH 8.6)
   5. 20 mM L-Leucine: Dissolve 54 mg leucine in 20 ml of 50 mM Tris/HCl buffer (pH 8.6).

B. Assay procedure.
   1. To each tube, add the following:
      50 µl of 50 mM Tris/HCl buffer (pH 8.6)
      10 µl of 1.6 mM Pyridoxal phosphate
      20 µl of 50 mM α-ketoglutarate
      100 µl of 20 mM Leucine

   (2 Blanks for Leucine for each tissue: all the above + 20 µl of 1.5 M $HClO_4$ + 20 µl tissue extract)

   2. Add 20 µl tissue extract to the 1.5 ml sample tube.
   3. Cap the tube. Place the tubes in a 26 °C water bath.
   4. After a 20 min in cubation period, add 20 µl 1.5 M $HClO_4$ into the incubation medium.
   5. To all tubes (including the samples and blanks):
      Add 10 µl of 2 M K_2CO_3. Mix. Centrifuge in a Microcentrifuge for 1 min. Use the supernatant fluid for glutamate analysis by HPLC.

Determination of hexokinase (HK) activity

A. Chemicals:
   1. 50 mM Tris-HCl/13.3 mM $MgCl_2$ buffer, pH 8.0: Dissolve 302.9 mg Tris-HCl (mw: 121.14) and 63.3 mg $MgCl_2$ in 45 ml H2O, use 1 N NaOH to adjust pH to 8.0, then make it to 50 ml with H_2O.
   2. 0.67 M Glucose: Dissolve 1.207 g D-glucose (mw: 180.156) in 10 ml Tris·$MgCl_2$ buffer.
   3. 16.5 mM ATP: 10 mg ATP-Na_2·$3H_2O$ (mw: 551.14 (anhydrous basis)) in 1 ml Tris·$MgCl_2$ buffer. (Prepare fresh)
   4. 6.8 mM NAD: 3.8 mg NAD (mw: 551.14 (anhydrous basis)) in 1 ml Tris·$MgCl_2$ buffer. (Prepare fresh)
   5. Glucose-6-phosphate dehydrogenase (G6PDD) solution, 300 U/ml in Tris·MgCl2 buffer. Prepare fresh

B. Assay Procedure:
   1. Prepare the following reaction mixture and prewarm :

Tris·$MgCl_2$ buffer, pH 8.0	36.5 ml
Glucose solution	8 ml
ATP solution	1.6 ml
NDA solution	1.6 ml
G6PDD solution	160 µl

   2. Add 200 ul sample into 96 well plate.
   3. Add 10 ul samples to each well.
   4. Measure the absorbance at 340 nm every 1 min for 5 min, at 25℃. For blanks, use 10 ul of 60 mM Tris·$MgCl_2$ buffer (pH 8.0) to replace 10 µl sample.

Determination of phosphofructokinase -1 (PFK-1) activity

A. Chemicals:
   1. 100 mM Tris buffer (pH 9.0): Dissolve 2.423 g Tris (base) in 180 ml H_2O. Adjust pH to 9.0 with 6 N HCl, and make up to a final volume of 200 ml with H_2O.
   2. 100 mM ATP: Dissolve 0.605 g ATP-Na2·3H_2O in 8.2 mL H_2O + 1.8 ml 1 N NaOH.
   3. 56 mM Phosphoenolpyruvate (PEP): Dissolve 0.150 g PEP MCA salt in 10 ml H_2O.
   4. 13.1 mM NADH: 0.1 g NADH-Na2·3H_2O in 10 ml H_2O.
   5. 500 mM Fructose 6-phosphate (F6P) ; Dissolve 1.55 g F6P disodium salt in 10 ml H_2O.
   6. 2.5 M KCI: 16.64g KCl in 100 ml H_2O.
   7. 100 mM MgSO4: 2.47 g MgSO4·7H_2O in 100 ml H_2O.
   8. Pyruvate kinase (PK): Dissolve PK in 1% BSA solution approx. 200 U/ml at 25°C.
   9. Lactate dehydrogenase (LDH): Dilute LDH at approx. 5,000 U/ml at 25 °C with Tris buffer.

B. Assay Procedure
   1. Prepare the following reaction mixture and pipette 3.00 ml reaction mixture into a cuvette.

Solution 1	27.33ml	Solution 6	0.06ml
Solution 2	0.30ml	Solution 7	0.60ml
Solution 3	0.39ml	Solution 8	0.06ml
Solution 4	0.60ml	Solution 9	0.06ml
Solution 5	0.60ml		

   2. Incubate at 30 °C for about 3 minutes.

   3. Add 0.01 ml of enzyme solution into the cuvette and mix.

   4. Read absorbance change at 340 nm per minute (ΔAbs340) in the linear portion of curve.

Determination of pyruvate kinase (PK) activity

A. Chemicals
   1. 0.06 M Tris-HCl buffer, pH 7.5: Dissolve 363.4 mg Tris-HCl (mw: 121.14) in 45 ml H_2O, use 1 N NaOH to adjust pH to 7.5, then make it to 50 ml with H_2O.
   2. 0.12 M $MgCl_2$: Dissolve 11.5 mg $MgCl_2$(mw: 95.211) in 1 ml H_2O.
   3. 2.25 M KCl: Dissolve 167.7 mg KCl (mw: 74.55) in 1 ml H_2O.
   4. 6 mM ADP: 2.83 mg ADP-$Na_2 \cdot 2H_2O$ (mw: 471.16) in 1 ml buffer. (Prepare fresh)
   5. 43 mM Phosphoenolpyruvate (PEP): 11.5 mg PEP MCA salt(mw: 267.22) in 1 ml Tris-HCl buffer. (Prepare fresh)
   6. 6 mM NADH: 4.6 mg NADH-$Na_2 \cdot 3H_2O$ (mw: 709.40 (anhydrous basis)) in 1 ml Tris buffer. (Prepare fresh)
   7. Lactate dehydrogenase (LDH) solution, 40 U/ml in buffer. (Prepare fresh)

B. Assay Procedure
   1. Prepare the following reaction mixture and prewarm :

Tris-HCl buffer, pH 7.5	24 ml
$MgCl_2$ solution	1.0 ml
KCl solution	1.0 ml
ADP solution	1.0 ml
PEP solution	1.0 ml
NADH solution	1.0 ml
LDH solution	50 µl

   2. Add 200 ul sample into 96 well plate.
   3. Add 10 ul samples to each well.
   4. Measure absorbance at 340 nm every 1 min for 5 min, at 25°C. For blanks, use 10 ul of 60 mM Tris-HCl buffer (pH 7.5) to replace 10 ul sample.

Determination of Carnitine Palmitoyltransferase I (CPT-I) activity assay

A. Chemicals:
   1. 20 mM Palmitoyl-CoA [MW: 1005.94]: Dissolve 1 g in 50 ml DD water. The stock solution can be stored at -20°C for several weeks and is stable upon freeze/thaw.
   2. 100 mM L-carnitine [MW: 161.199]: Dissolve 162 mg in 10 ml DD water. Store at -20°C.
   3. L-[N-methyl-^{14}C] carnitine-HCl is obtained from American Radiolabeled Chemicals (ARC 308) at 0.1 mCi/ml in 50 % EtOH and stored at 4°C.
   4. 1.2 M HCl: Add 5 ml 6 N HCl in to 20 ml DD water.
   5. 150 mM KCl [MW: 74.5513]/5 mM Tris-HCl [MW: 157.60], pH 7.2: Dissolve 560 mg KCl, 40 mg Tris-HCl in 45 ml DD water. Adjust pH with HCl and make it to 50 ml by DD water, then store at 4°C.
   6. 4 M MgCl2 [MW: 95.211] : Dissolve 3.808 g $MgCl_2$ in 10 ml DD water. Stable at room temperature.
   7. Rotenone [MW: 394.41] (Sigma, R-8875): Dissolve 40 mg in 1 ml acetone. Store in a
      tightly capped tube at 4°C. Discard after 1 month.
   8. 210 mM Tris-HCl [MW: 157.60], pH 7.2: Dissolve 1.66 g Tris-HCl in 45 ml DD water. Adjust pH with HCl and make it to 50 ml by DD water. Store at 4°C.
   9. 1-Butanol.
   10. Solid reagents: bovine serum albumin, essentially fatty acid-free.
   11. Adenosine triphosphate (ATP). (Sigma. A-2283).
   12. KCN [MW: 65.12] (Aldrich, 20,781-0). Note, KCN is highly toxic and releases hydrogen cyanide gas when in contact with acid. Perform this in the hood. Gloves should be worn when handling KCN and caution should be observed when storing.
   13. Substrate Mix (5x): To 4.75 ml water, add the following:
       62.5 µl of 20 mM palmitoyl-CoA
       125 µl of unlabeled 100 mM L-carnitine
       62.5 µl of [^{14}C]carnitine.

   [Final concentrations in the reaction solution are 50 µM palmitoyl-CoA, 500 µM carnitine and 0.25 µCi/ml [^{14}C]carnitine (see Note 3). Substrate Mix can be stored at −20°C for several weeks
   and can be frozen and thawed several times without harm (see Note 4).]

   14. Assay Cocktail (2x): To 50 ml of 210 mM Tris-HCl, add:

       1 g bovine serum albumin (BSA) (mix gently)
       Then, add the following:
       203 mg ATP
       7.7 mg glutathione
       13 mg KCN (as solids)
       100 ul of 4 M MgCl2
       100 µl of 40 mg/ml rotenone

[Final concentrations: 210 mM Tris-HCl (pH 7.2), 2 % (w/v) bovine serum albumin, 8 mM ATP[MW: 507.18], 8 mM MgCl2, 0.5 mM glutathione [MW: 307.32] (reduced form), 80 µg/ml (w/v) rotenone and 4 mM KCN (see Note 5).]

Note, the buffer will turn cloudy on addition of the rotenone, but will clear on continued stirring. Cocktail must be prepared fresh daily.

B. Assay Procedure:
   1. Add the following to 2 ml plastic tubes:

      100 µl of 5x substrate mix,
      50 µl of 150 mM KCl/5 mM Tris, pH 7.2,
      250 µl of 2x Assay Cocktail.

[Final concentrations of substrates during the reaction are 50 µM palmitoyl-CoA and 500 µM [^{14}C]carnitine.]

   2. Add 100 µl of enzyme sample. Vortex briefly.
   3. Place the tubes in a 26°C shaking water bath.
   4. After 15 min, add 500 µl of 1.2 M HCl and vortex.
   5. Add 500 µl of 1-butanol. Vortex each tube for 30 s.
   6. Centrifuge at 10,000 g for 1 min. Obtain the upper butanol phase.
   7. Transfer 300 µl of the upper, butanol phase to a 1.5-ml microfuge tube containing 500 µl of water. Cap the tubes and vortex the tube. Repeat Step 6.
   8. Transfer 250 µl of the upper phase to a scintillation vial. Add 5 ml Aqueous cocktail for counting.

Note: For blanks,add:

100 µl of 5x substrate mix,
50 µl of 150 mM KCl/5 mM Tris, pH 7.2,
250 µl of 2x Assay Cocktail.
500 µl 1.2 N HCl and vortex.

Add 100 µl of enzyme sample. Wait 5 min.

Repeat steps 3-8 above.

APPENDIX B

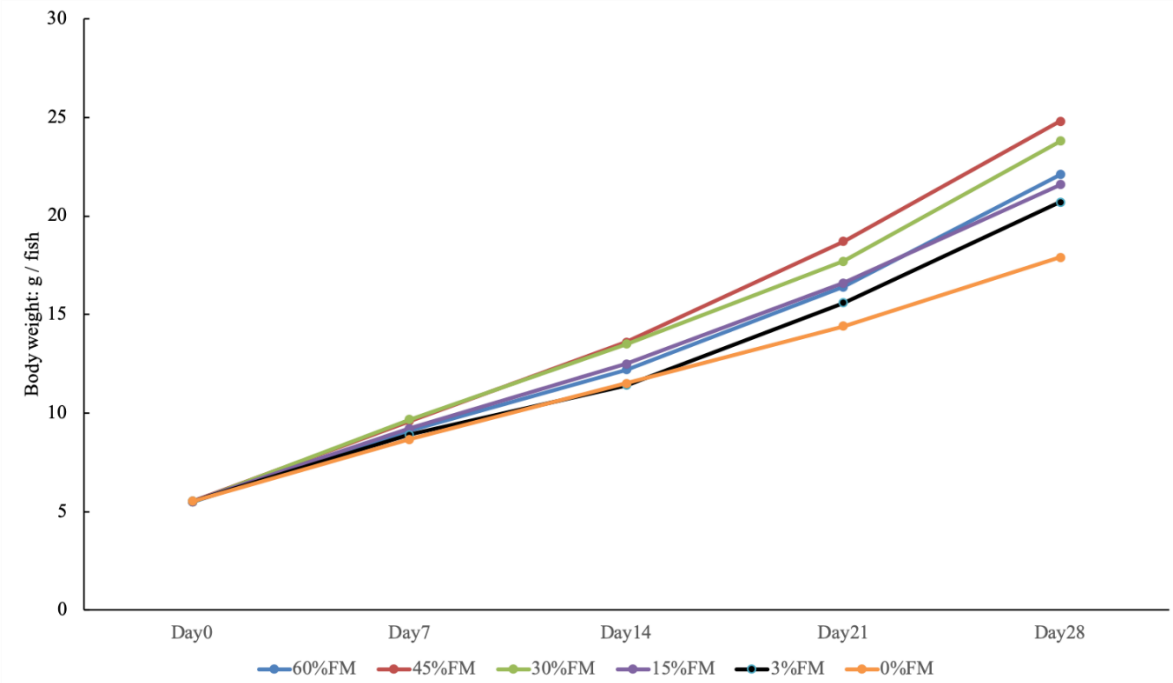

Figure B.1 Growth of body weight of HSB that were fed the experimental diets for four weeks

Table B.1 Custom DMEM Formulation

Components	**g/L**
(1) Inorganic salts	
$CaCl_2$•$2H_2O$	0.265
$Fe(NO_3)_3$•$9H_2O$	0.0001
$MgSO_4$	0.09767
KCl	0.4
$NaHCO_3$	3.7
NaCl	6.4
NaH_2PO_4	0.109
Succinic Acid	-
Sodium Succinate	-
(2) Amino acids	
L-Arginine•HCl (0 µM)	**0**
L-Cystine•2HCl (0 µM)	**0**
L-Glutamine (0 µM)	**0**
Glycine (0 µM)	**0**
L-Histidine•HCl•H_2O (100 µM)	0.021
L-Isoleucine (150 µM)	0.020
L-Leucine (0 µM)	**0**
L-Lysine•HCl (200 µM)	0.036
L-Methionine (75 µM)	0.012
L-Phenylalanine (100 µM)	0.017
L-Proline (0 µM)	**0**
L-Serine (200 µM)	0.021
L-Threonine (200 µM)	0.016
L-Tryptophan (75 µM)	0.020
L-Tyrosine (free base)	-
L-Tyrosine •2Na•$2H_2O$ (100 µM)	0.026
L-Valine	**0**
(3) Vitamins	
Choline Bitartrate	-
Choline Chloride	0.004
Folic Acid	0.004
Myo-Inositol	0.0072
Niacinamide	0.004
D-Pantothenic Acid•1/2 Ca	0.004
Pyridoxal•HCl	-
Pyridoxine•HCl	0.004
Riboflavin	0.0004
Thiamine•HCl	0.004
(4) Others	
D-Glucose (0 µM)	**0**
HEPES	**0**
Phenol Red•Na	**-**
Sodium Pyruvate (100 µM)	0.011
Alanine (350 µM)	0.031
Aspartic acid (20 µM)	0.003
Asparagine (50 µM), Anhydrous	0.007
Glutamic acid (75 µM)	0.011
Taurine (100 µM)	0.013

To prepare 0.5 L solution (basal medium), add the following chemicals to 448 ml DD-water:

3.8 g powder (solid culture-medium ingredients)
1.85 g $NaHCO_3$
5 ml Antibiotics (P/S)

L-Arginine-HCl (200 µM):	21 mg	
L-Cystine-2HCl (75 µM):	12 mg	(or 9 mg L-cysteine)
L-Glutamine (500 µM):	37 mg	
Glycine (250 µM):	10 mg	
L-Leucine (250 µM):	17 mg	
L-Proline (250 µM):	15 mg	
L-Valine (250 µM):	15 mg	
D-Glucose (5 mM):	450 mg	
Hepes (20 mM, pH 7.4):	25 ml of 400 mM Hepes (pH 7.4)	

Adjust pH to 7.2 with 2 ml of 1 M HCl. Add 50 ml H_2O. Filter the solution through 0.2-µm filter.