DIET DEVELOPMENT FOR THE LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) JUVENILES

A Dissertation

by

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ABSTRACT

A series of *in vitro* and *in vivo* studies were conducted to determine: (1) the relative importance of amino acids (AAs), fatty acids and glucose as metabolic fuels for tissues of largemouth bass (LMB, a carnivorous fish); and (2) the optimal requirements of LMB for dietary protein, lipids and starch; and (3) the feasibility of replacing dietary fishmeal with alternative animal- and plant-source protein feedstuffs. Results of the first study (Chapter II) indicated that glutamate, glutamine and aspartate were oxidized at higher rates than glucose and palmitate for ATP production in the proximal intestine, liver, kidneys, and skeletal muscle of LMB. All the tissues had high activities of phosphate-activated glutaminase, glutamate dehydrogenase, and glutamate transaminases. Glutamine, glutamate and aspartate together generated 60-70% of ATP in LMB tissues and supported most of their oxygen consumption. Findings from the second study (Chapter III) demonstrated that the rates of glutamate and glutamine oxidation in these tissues were regulated by dietary protein intake in a tissue-specific manner to meet their metabolic needs. The fish had particularly high requirements for dietary protein to primarily satisfy their needs for glutamate, glutamine and aspartate as energy sources and minimize the hepatic accumulation of glycogen. Feeding experiemnts revealed that increasing the dietary crude protein content from 40% to 50% improved the growth performance and liver morphology of LMB (Chapter VI) and that excess dietary levels of starch (≥ 10%) resulted in hepatic glycogenosis (Chapter V). This metabolic disorder was due to the limited utilization of glucose by tissues and prolonged elevations of blood glucose for glycogen synthesis in the liver. Based on growth, metabolic and histologic data, we

recommend dietary crude protein (CP), lipids and starch levels to be 45%, 10% and < 10%, respectively, for juvenile LMB. Data from Chapter VI showed that 15% fishmeal protein (dry matter basis) in the diet could be replaced by low-cost poultry by-product meal plus soybean meal without affecting growth rates and feed efficiency of LMB. However, this practice resulted in inadequate provison of methionine and taurine, and black skin syndrome characterized by not only black spots in the skin but also damage in the eye, intestine and liver. In the final experiment (Chapter VI), supplementation with 0.5% methionine to low fishmeal diets improved the growth performance, feed efficiency, and health of LMB, while reducing the incidence of black skin syndrome in the fish from 38% to 10%. Collectively, findings from the current study not only advance basic knowledge on the nutrition, metabolism and functions of AAs in fish species, but also provide data for developing next generation of practical feeds for LMB to improve the efficiency of their growth and sustain their global production.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Guoyao Wu (Chair), as well as Dr. Stephen B. Smith (Department of Animal Science, College of Agriculture and Life Sciences), Dr. Gregory A. Johnson (Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences), and Dr. Duncan MacKenzie (Department of Biology, College of Science). All work for the dissertation was completed independently by Mr. Xinyu Li with technical assistance of Dr. Gayan I. Nawaratna (Department of Animal Science).

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NOMENCLATURE

AA	Amino acid
ALT	Alanine transaminase
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acid
BCKA	Branched-chain α-ketoacid
CP	Crude protein
FI	Feed intake
FCR	Feed conversion ratio
HSI	Hepatosomatic index
IPFR	Intraperitoneal fat ratio
KHB	Krebs-Henseleit bicarbonate
LMB	Largemouth bass
LDH	Lactate dehydrogenase
MS-222	Tricaine methanesulfonate
PER	Protein efficiency ratio
VSI	Viscerosomatic index
WG	Weight gain
α-KG	α-ketoglutarate

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background

Aquaculture is the fastest growing agricultural enterprise to provide high-quality animal protein for human consumption. About 70% of global aquaculture (excluding aquatic plants) relies on commercially compounded feed that is produced by mixing defined feed ingredients (Béné et al. 2016). Whereas some fish species may have a better efficiency of feed utilization for growth than land animals (Fig I-1 A), protein retention in growing fish as the percentage of dietary protein intake is similar to and even lower than that in growing pigs or chickens (Figs I-1B, 1C). Interestingly, fish generally have 2- to 3-fold greater requirements for dietary protein (30% to 55%) than livestock species (12% to 20%; Wu 2018). High levels of protein in diets increase nitrogen excretion, which is eventually discharged from aquaculture production systems to cause environmental pollution. To date, fishmeal is the most important protein source for fish in aquaculture and is derived from wild-harvested whole fish or shellfish. It has been reported that 20 million tons of fish are used for fishmeal production each year, and about 70 % of this is directed towards aquaculture, followed by the pig and chicken industries (Cashion et al. 2017). However, 90 % of the fish currently used for fishmeal production could be consumed by humans (Cashion et al. 2017). Therefore, although aquaculture has benefits on economic development and food provision, public debate on this agricultural sector is dominated by concerns over resource utilization and environmental sustainability (Béné et al. 2016). It is thus imperative to understand why fish have particularly high requirements for dietary proteins, with the objective being to maximize the efficiency of protein utilization by fish and reduce the use of fishmeal in aquaculture.

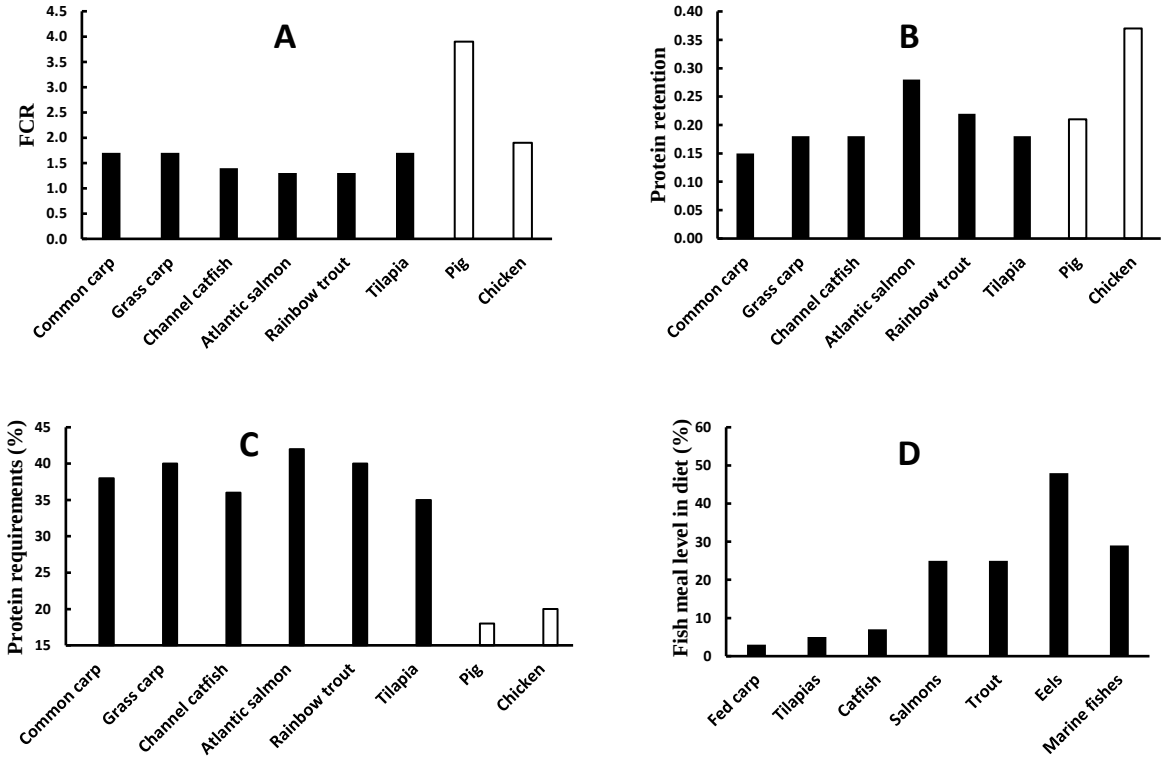


Figure I-1 A. The average of FCR (feed conversion rate, dry feed intake / weight gain) from different animals. Source: Adapted from Fry et al. (2018); B. The average of protein retention in edible portion from different animals. Source: Adapted from Fry et al. (2018); C. The protein requirements of different animals. Source: Adapted from Wilson et al. (1986), Kim et al. (1991), Shelton (1971), Applegate & Angel (2008); D. fishmeal inclusion in compound aquafeed of different fish species and species groups. Source: Adapted from FAO (2012).

Amino acids (AAs) play important roles in fish nutrition by serving as the building blocks of protein and precursors of low-molecular-weight substances (e.g., NO, creatine, polyamines, GABA, catecholamines, and glutathione) with enormous physiological importance, as well as by regulating key metabolic pathways that are vital to the growth, development, reproduction. Besides, AAs participate in biological oxidation, gluconeogenesis and lipogenesis in a cell- and tissue-specific manner. The elevated requirements of fish for dietary protein and AAs may be essential for growth, immune responses, antioxidant reactions, metamorphosis, and adaptations to

environmental changes in fish species. Understanding the metabolism and functions of AAs is fundamental for manufacturing environmentally-friendly aquafeeds and reducing dietary protein levels and feed costs in fish production.

Metabolism of AAs

Oxidation

In view of animal production, the most important role of AAs is to serve as the building blocks of proteins (Wu 2018). However, most of fish species are carnivorous which use primarily AAs as energy substrates to provide ATP (Ballantyne 2001). For example, 35-40% of leucine is oxidized for ATP production in fish (Fauconneau and Arnal 1985). Likewise, the oxidation of AAs as an entity may contribute to 50–70% of total energy needs in the marine fish embryos and yolk-sac larvae (Rønnestad and Fyhn 1993; Rønnestad et al. 1999). We have recently shown that glutamate plus glutamine plus leucine contributes to ~80% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle of zebrafish (Jia et al. 2017) and hybrid striped bass (Jia et al. 2017). Individual AAs have their own catabolic pathways because of their different structures. However, the catabolism of many AAs shares a number of common steps to generate pyruvate, oxaloacetate, α-ketoglutarate (α-KG), fumarate, succinyl-CoA, and acetyl-CoA. For example, the carbon backbones of some AAs are converted to α-KG by glutamate dehydrogenase and transaminases (Li et al. 2020). Glutamate dehydrogenase is also quantitatively importent for glutamate and glutamine catabolism in fish (Wilson 1973; Ballantyne 2001). Alanine transaminase (ALT) and aspartate transaminase (AST) play an important role in initiating the degradation of alanine and aspartate to yield pyruvate and oxaloacetate, respectively (Wu 2013b). For the catabolism of leucine in mammals, it undergoes active transamination with α-KG to form α-ketoisocaproic acid

and glutamate primarily in skeletal muscle. Then, the α-ketoisocaproic acid is converted into acetyl-CoA by the branched-chain α-ketoacid (BCKA) dehydrogenase complex primarily in the liver (Wu 2013b). Little is known about the inter-organ metabolism of leucine and other branched-chain AAs in fish. Results of our recent studies indicated that leucine was extensively transaminated and decarboxylated in the liver of LMB (our unpublished work). It is therefore possible that patterns of the catabolism of branched-chain AAs differ between fish and land mammals.

Gluconeogenesis and lipogenesis

Although AAs are important metabolic fuels in carnivorous fish species, glucose is still required for aerobic oxidation in the nervous system and certain other cell types (e.g., red blood cells) and as a precursor for the syntheses of glycogen and mucopolysaccharides (Bever et al. 1981). Most AAs are quantitatively important glucogenic substrates in fish, which consume only a small amount of dietary carbohydrate in their lives (Cowey et al. 1977; Bever et al. 1981). The most important AAs for gluconeogenesis in the fish liver appear to be alanine, glutamate, serine, and aspartate. For gluconeogenesis, there are mainly four unidirectional rate-limiting steps catalyzed by the enzymes pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase. Gluconeogenesis from alanine or glutamate is increased by fasting in kelp bass (*Paralabrax sp*.; Bever et al. 1981). In another study, the whole-body synthesis of glucose from [U-^{14}C] glutamate was markedly increased by the low-carbohydrate diet or starvation in rainbow trout (*Salmo gairdneri*; Cardenas 1985). During prolonged starvation plus exercise, the rates of gluconeogenesis from AAs increased two-fold and, simultaneously there was a corresponding increase in PEPCK activity in the liver of rainbow trout

(French et al. 1981). All of these results support the view that the primary function of gluconeogenesis from AAs is to meet the needs of the body for glucose when dietary carbohydrate intake is inadequate. Exogenous glucose and AAs could produce acetyl-CoA, which could increase fatty acid (FA) synthesis in fish. Interestingly, de novo lipogenesis from glucose in the liver is limited in some fish, especially when they have low carbohydrate intake (Jürss and Bastrop 1995). AAs are the better precursors for lipid synthesis compared to glucose in some fish species (Nagai and Ikeda 1972, 1973). For example, in juvenile carp (*Cyprinus carpio*), glutamate was preferentially incorporated into hepatopancreatic lipids than glycogen (Nagai and Ikeda 1972).

Derivatives of AAs

AAs serve as substrates for the synthesis of many substances with enormous physiological importance (Wu 2013b). These metabolites are essential for the health, growth, and development of animals. For example, GABA is synthesized from glutamate, which is the major inhibitory neurotransmitter in the central nervous system (Wagner et al. 1997) and plays an important role in the control of pituitary hormone secretion, anoxic metabolic depression, sex steroidal regulation and excitatory responses (Nilsson 1992; Lariviere et al. 2005). Carnosine (β-alanyl-L-histidine), with a characteristic imidazole-ring, is a dipeptide molecule, made up of β-alanine and histidine. Carnosine is an antioxidant and important buffer in the skeletal muscle of aquatic animals, especially migratory pelagic marine fishes (Snyder et al. 2012). Glutathione (L-glutamyl-L-cysteinyl-glycine, GSH) is a tripeptide formed from glycine, cysteine, and glutamate. Glutathione is capable of protecting cellular components from damage by reactive oxygen species, such as free radicals, peroxides, lipid peroxides, and heavy metals in fish species (Peña-Llopis et al. 2003).

The functions of dietary AAs in fish

Survival, growth and muscle development

Protein is an essential component for every cell in the body and undergoes continuous turnover (synthesis and degradation). AAs not only serve as the building blocks of protein but also play an essential role in whole-body homeostasis (Wu 2013a,b). At present, the NRC (2011) recommends dietary EAA requirements for fish, but does not provide any values for nutritionally nonessential AAs (NEAAs), including glutamate and glutamine. However, using a chemically purified diet that provides all nutritionally essential AAs (EAAs) in NRC (2011)-recommended amounts, all NEAAs but no glutamate and glutamine, as well as sufficient amounts of fatty acids, carbohydrate, minerals and vitamins, juvenile hybrid-striped bass (HSB) grew poorly in comparison with fish fed the purified diet containing no glutamate or glutamine (Jia et al. 2019). Beginning on Day 18 of the experiment, deaths of the fish occurred in all tanks of fish fed the purified diet without glutamate or glutamine. By Day 35 of the experiment, survival rates in the different treatment groups of the juvenile HSB were as follows: the 60% fishmeal diet, 97%; the complete purified diet (containing all AAs), 89%; the purified diet without glutamate, 39%; the purified diet without glutamine, 39% (Jia et al. 2019). These results indicate that the endogenous synthesis of glutamate or glutamine is insufficient for the growth or survival of the HSB and that these two AAs are nutritionally essential for the fish.

In growing fish, protein synthesis exceeds protein degradation, resulting in protein deposition (NRC 2011). Intracellular protein synthesis requires AAs and energy supply. Traditionally, the requirements for dietary AAs were determined based on the growth performance or protein deposition in fish fed different levels of a given AA. It should be borne in mind that protein

deposition is the main determinant of body weight gain in growing fish (Dumas et al. 2007). As in other animals, fish need 20 different proteinogenic AAs to synthesize protein. About 25 to 55% of dietary AAs are used for protein accretion in growing fish (NRC 2011). Generally, the rates of lean tissue gain and protein retention in fish increase progressively when the content of protein or AAs in the diet increases from a suboptimal to an optimal level, beyond which the rates of lean tissue gain and protein retention either remains at the plateau or declines.

Fish continue to grow throughout their lives, but the relative growth rate (%/day) decreases with age. Both hyperplasia (increases in fiber number) and hypertrophy (increases in fiber size) contribute to adult myotomal muscle growth for fish (Johnston 2001). Skeletal muscle formation or myogenesis involves the specific control of several myogenic regulatory factors (MRFs) which control a series of events, including the specification, activation, and differentiation of myogenic cells. The maintenance of formed muscle fibers is dependent on a balance between protein synthesis and protein degradation (Fuentes et al. 2013). The mechanistic target of rapamycin (MTOR) plays a key role in cell physiology, acting primarily at the initiation of polypeptide synthesis (Wang et al. 2006; Duan et al. 2015). The pathway can be directly activated by intracellular AAs through the mediators of Rag, GTPase, Rheb, hVps34, and MAP4K3 (Duan et al. 2015). Dietary AAs, such as leucine, glycine, glutamine, and arginine are capable of regulating the MTOR signaling pathway in fish species (Chen et al. 2015; Liang et al. 2018; Li et al. 2019a). For example, increasing dietary levels of leucine enhanced MTOR expression, growth performance and whole-body protein gain in juvenile blunt snout bream (Liang et al. 2018). Myostatin is a negative regulator of myogenesis, and its mRNA expression in fish could be suppressed by proper supplementation with histidine to the diet (Michelato et al. 2017). Glutamate

and glutamine are important in the growth of proliferating muscle cells as well as the acceptable firmness and quality of fish fillets (Østbye et al. 2018; Ingebrigtsen et al. 2014). The effects of these two AAs may be mediated, in part, through activating the MTOR pathway.

Secretion of hormones

AAs regulate muscle growth and development through direct actions on myogenic regulatory factors and MTOR signaling, or indirectly via the growth hormone (GH)/insulin-like growth factor (IGF) axis (Vélez et al. 2017). As in other animal species, the GH/IGF axis plays an important role in muscle protein synthesis as well as muscle cell growth through both hyperplasia and hypertrophy. GH can exert a direct effect on the muscle or indirectly through IGF-I secreted by the liver. IGF-1 modulates cell metabolism (e.g. nutrient uptake) and the MTOR signaling pathway, which controls both protein turnover and muscle cell proliferation. Previous studies have demonstrated that there is cross-talk between ghrelin and neurotransmitters, such as AAs and serotonin, to regulate GH secretion (Pinilla et al. 2003). In humans, AA intake increased ghrelin secretion to further stimulate the GH/IGF axis (Knerr et al. 2003). Dietary AAs and protein are also important nutrients that positively influence the GH-IGFs axis in fish (Picha et al. 2008). For example, Bower and Johnston (2010) have shown that AAs can enhance the expression of many genes in the IGF signaling pathway in Atlantic salmon. In another study, a deficiency of AAs, especially lysine, affects the expression of genes in the IGF system and of myogenic factors in gilthead sea bream (Azizi et al. 2016). In rainbow trout, dietary methionine could increase the expression of genes involved in the GH/IGF axis response and protein turnover (Rolland et al. 2015). More details about the effects of AAs on GH/IGF axis and muscle development in different fish species are presented in Fig I-2.

Cholecystokinin (CCK) plays an important role in controlling digestion in vertebrates (Wu 2018). In humans, the most potent stimulants of CCK secretion are the partially digested products of fat and protein, including di- and tri-peptides (Liddle 2000). In sea bass larvae, different levels of protein or its hydrolysate in diets modulate trypsin expression and affect CCK content (Cahu et al. 2004). There are reports that ingestion of liposomes containing free AAs, protein or their combinations effectively stimulates CCK production in first-feeding herring larvae (Koven et al. 2002). In mammals, the secretion of other hormones [e.g., insulin, gonadotropin-releasing hormone (GnRH) and cortisol] may also be affected by intakes of dietary protein and AAs (Bourguignon et al. 1989; Kraemer et al. 2006; Veldhorst et al. 2019). However, such studies are limited in fish species.

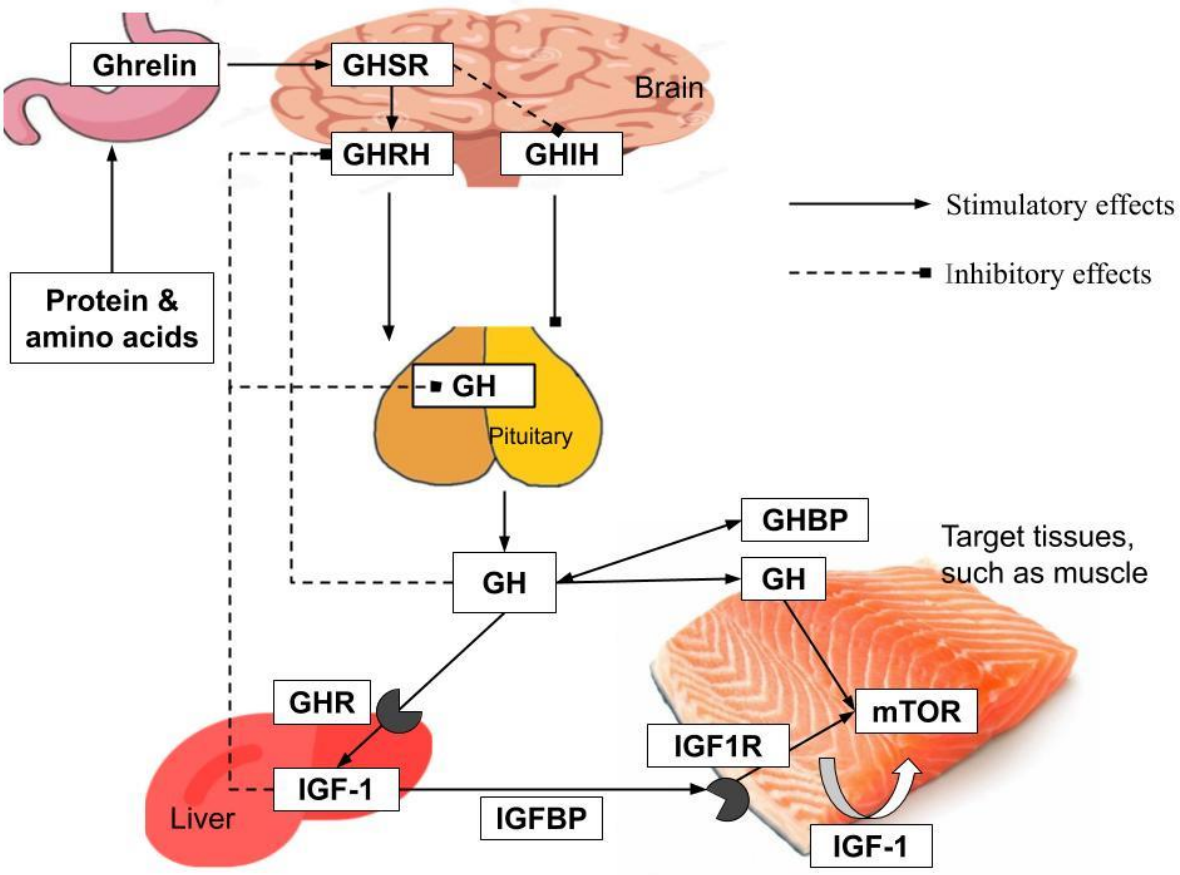


Figure I-2 Illustration of the endocrine axis (GH/IGF axis) that regulates the growth of teleost fish. GH, growth hormone; GH, growth hormone receptor; GHBP, growth hormone-binding protein; GHRH, growth hormone-releasing hormone; GHIH, growth hormone-inhibiting hormone; GHSR, growth hormone secretagogue receptor; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor; MTOR, mechanistic target of rapamycin; IGFBP, insulin-like growth factor-binding protein.

Attractants

The use of AAs as dietary attractants has received considerable attention because the replacement of fishmeal with plant-source protein feedstuffs often reduces the feed intake of aquatic animals. Vertebrates express two families of G-protein-coupled receptors (GPCRs), T1Rs and T2Rs, in their taste buds (Oike et al. 2007). In mammals, the heteromeric T1R1/3 receptor

responds to umami tastants such as glutamate and nucleic acids, whereas the T1R2/3 responds to sweet tastants such as sugars (Oike et al. 2007; Yarmolinsky et al. 2009). The T2Rs respond to bitter tastants, including poisonous chemicals (Chandrashekar et al. 2000; Mueller et al. 2005). However, both T1R1/3 and T1R2/3s act as receptors for AAs but not sugars in fish (Oike et al. 2007). The facial nerve of zebrafish responds strongly to alanine and proline; moderately to cysteine, glycine, serine, tyrosine, quinine HCl, and denatonium; and weakly to other AAs. Glycine and some L-AAs (e.g., alanine, glutamate, arginine, and glycine) possess dietary attractant properties, which can trigger reflexive snapping and biting behaviors (Kasumyan and Morsi 1996; Polat and Beklevik 1999; Derby and Sorensen 2008). Similarly, Shamushaki et al. (2007) reported that alanine and glycine are potent attractants for Persian sturgeon juveniles. Some non-proteinogenic AAs (e.g., D-glutamine, D-asparagine, D-glutamate, and β-alanine) are also strong attractants for glass eels (Sola and Tongiorgi 1998). DL-alanine is also an attractant that has a very strong effect on improving the survival or growth of post-larval African catfish (*Clarias gariepinus*; Yilmaz 2005) and juvenile Sea Bass (*Dicentrarchus labrax*; Tekelioglu et al. 2003). As a result, marine by-products (such as krill shrimp meal) that contain comparatively balanced AAs and high amounts of free AAs, nucleotides, and nucleosides can improve the feed intake and growth of fish fed low-fishmeal diets (Kader et al 2012).

Immune responses

Immunity is the ability of an organism to resist attacks by pathogens. Generally, there are three levels of immune defense in fish. The first line consists of physical and epithelial barriers such as scales, skin, mucus, gastric acid, and chemical mediators [such as lysozyme, transferrin, complement systems, reactive oxygen species (ROS), and reactive nitrogen species]. The second

line of defense involves cells, including phagocytes, natural cytotoxic cells (NCC), and inflammatory response. The third line of defense is the development of a specific immune response through the production of antibodies by B-cells against specific pathogens or the development of T-cell responses (Trichet 2010; Webster et al. 2015). The nutritional status of the host can influence the severity of impacts from pathogens (e.g., viruses, bacteria, fungi, and parasites) as well as immunity acquisition (Webster et al. 2015). AAs hold great promise in improving health and preventing infectious diseases in animals (including fish and shrimp) and humans (Li et al. 2007).

AAs play fundamental roles in the immune system of fish (Li et al. 2009). In fish, glutamine is crucial to the immune response as it is a major energy substrate to support optimal lymphocyte proliferation and production of cytokines by lymphocytes and macrophages as reviewed previously (Alejo and Tafala 2011; Li et al. 2009; Reyes-Cerpa et al. 2012). Macrophage-mediated phagocytosis is influenced by glutamine availability (Calder and Yaqoob 1999). Glutamine is essential for proliferation of the T and B cell lymphocytes in fish, as dietary glutamine increased lymphocyte proliferation in channel catfish head-kidney and spleen (Pohlenz et al. 2012b). Results of *in vitro* studies have shown that arginine and glutamine are important immunomodulators of both innate and adaptive responses in fish leukocytes (Pohlenz et al. 2012 a, b). In recent years, the positive function of dietary glutamine on the immune responses has been well studied in several fish species. Arginine is an abundant AA in tissue proteins and plays an important role in the immunity of the host directly through the production of NO and polyamines by macrophages, or indirectly via affecting gene expression and endocrine status (Li et al. 2009; Andersen et al. 2016). For example, NO is a cytotoxic molecule of macrophages and mediates inflammation (Wu, 2013 a,b). Both *in vivo* and *in vitro* experiments in channel catfish indicated that arginine has positive

effect on the immune system, as dietary arginine supplementation enhanced the pathogen-killing and phagocytosis abilities of macrophages (Buentello et al. 2007; Pohlenz et al. 2012 a, b). Higher serum lysozyme activity was observed in fish fed the diet supplemented with 1% arginine, 2% arginine, 1% glutamine, or 1% arginine plus 1% glutamine in hybrid striped bass (Cheng et al. 2012). Methionine has effects on the immune system by improving both cellular and humoral immune responses (Rubin et al. 2007). As noted previously, methionine is involved in polyamine and GSH syntheses, which may also affect the proliferation of lymphocytes and inflammatory processes in cells (Grimble and Grimble 1998). Some studies also reported that certain AAs, such as taurine and lysine, might modulate immune responses in aquatic animals (NRC, 2011). More studies are necessary to understand the complex relationship between AAs and the immune system.

Anti-oxidative defenses

Free radicals play a beneficial role in biological evolution, metabolism, and physiology, but pathological levels of these substances also have an adverse effect on oxidative damages to protein, lipids and DNA, leading to cell injury and death (Fang et al. 2002). The production and deleterious effects of free radicals are illustrated in Fig I-3. ROS, including superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2), contribute to radiation and oxidant-induced cytotoxicity. Fish species are highly susceptible to ROS as their tissues contain higher levels of poly-unsaturated fatty acids (PUFAs) than those in mammals and birds (Enser et al. 1996). To prevent these harmful effects, ROS should be rapidly removed by non-enzymatic and enzymatic antioxidants (Fang et al. 2002; Martinez-Alvarez et al. 2005). Glutathione peroxidase (GPx) acts to reduce lipid hydroperoxides to their corresponding alcohols and convert free H_2O_2 to H_2O. Thus,

this enzyme is crucial for efficient protection against lipid peroxidation. Besides GPx, catalase (CAT) and superoxide dismutase (SOD) are two other major antioxidant enzymes of the antioxidant defense system. SOD catalyzes the conversion of O_2^- to H_2O_2, which is further converted to H_2O by CAT (Fang et al. 2002).

AAs are important nutrients for anti-oxidative defense as they can be building blocks for the of synthesis antioxidant enzymes. Some AAs (arginine, citrulline, glycine, proline, 4-hydroxy-proline, taurine and histidine) can directly remove oxygen free radicals (Fang et al. 2002; Wu et al. 2019a). The antioxidant ability of fish could be improved by AAs, such as arginine (Liang et al. 2018b), glutamine (Zhu et al. 2011; Han et al. 2014b), taurine (Han et al. 2014b; Pino et al. 2010), methionine (Elmada et al. 2016), leucine (Deng et al. 2016), lysine (Li et al. 2014), histidine (Feng et al. 2013), citrulline (Li et al. 2013), and proline (Li et al. 2013). In most of those studies, authors made conclusions based on the activities and gene expression of antioxidant enzymes. In the study with juvenile yellow catfish, optimum dietary methionine decreased peroxidative damage in tissues, because SOD and GPX activities decreased with increasing dietary methionine levels (Elmada et al. 2016). Recovery from oxidative damage can be associated with a reduction in inflammatory molecules. Thus, changes in the expression or activities of antioxidative enzymes may reflect either an increase or decrease in oxidative stress. For this reason, the evaluation of effects of AAs on antioxidative responses should be carefully performed with fish under different conditions. Besides AAs, small peptides (GSH and carnosine) and nitrogenous metabolites (creatine) are also important compounds for scavenging oxygen free radicals (Wu 2013a,b; Li and Wu 2018). For example, dietary supplementation with L-carnitine elevated the levels of enzymatic antioxidants, such as SOD, CAT, glutathione S-transferase (GST) activities, in tissues of juvenile

black sea bream (Ma et al. 2008). Glutamate (derived from diet, synthesis, or glutamine hydrolysis), cysteine and glycine are required for the synthesis of GSH, thereby improving the repairing ability of enterocytes in fish (Hu et al. 2014).

In all animals, including fish and shrimp, the normal function of organs depends on their structural integrity, which can be affected by radiation-induced injury (Wen et al. 2014). In other words, an increase in antioxidant capacity brought about by dietary AAs is important for the health and growth of aquatic animals. Li et al. (2016) suggested that lysine plays a significant role in protecting the intestine of fish *in vivo* and *in vitro* through the induction of expression of key antioxidant genes. Similarly, Rimoldi et al. (2016) reported that taurine supplementation to soybean meal-based diets could increase the length of villi folds, reduce the number of vacuoles, and increase the number of goblet cells. Decreases in the length of villi and the number of goblet cells were observed in turbot (*Scophthalmus maximus* L.) fed methionine-deficient diets (Gao et al. 2019). Moreover, some traditionally nonessential AAs, such as glutamate and glutamine, could promote the antioxidant capacity in fish, which could further enhance intestinal development and growth (Li et al. 2013; Jiang et al. 2015). It should be noted that an improvement in antioxidant capacity is not the only variable for assessing the positive functions of AAs in different organs, as AAs are also major substrates for ATP production and essential for protein synthesis (Jia et al. 2017).

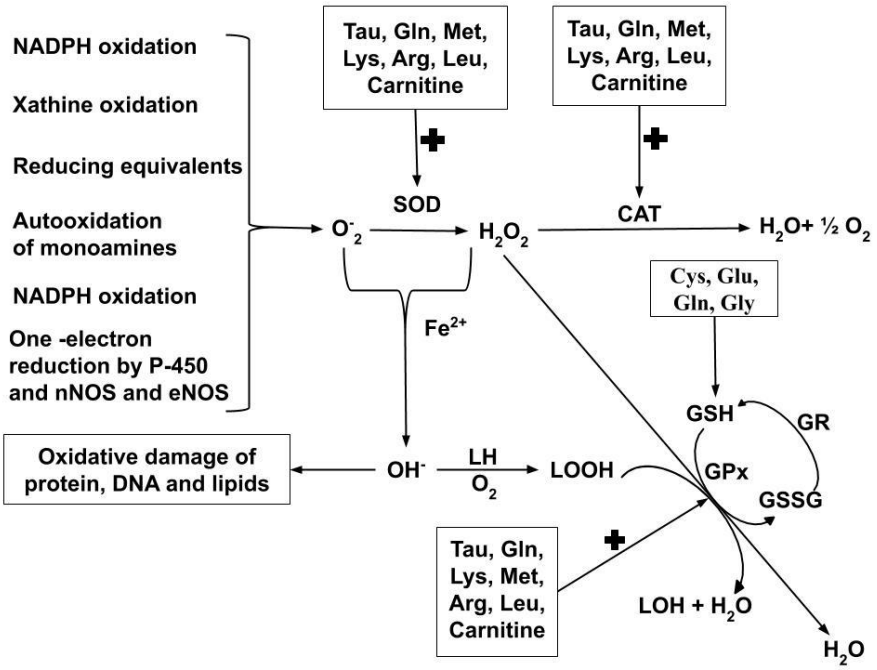


Figure I-3 The mechanisms whereby AAs and their metabolites serve as antioxidants in fish (adapted from Fang et al. 2002). Arg, arginine; Cys, cystine; CAT, catalase; Gln, glutamine; Glu, glutamate; Gly, glycine; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutahione; GSSG, oxidized glutathione; Lys, lysine; Leu, leucine; LH, lipids (unsaturated fatty acids); LOOH, lipid hydroperoxide; Met, methionine; SOD, superoxide dismutase; Tau, taurine.

Lipid digestion and metabolism

As in animals, lipid metabolism in fish includes digestion, absorption, transport, lipogenesis, and biological oxidation (primarily the mitochondrial β-oxidation) (Wu 2018). The release of CCK is stimulated by the entry of long-chain fatty acids into the stomach or duodenum of fish. CCK has two major functions in lipid digestion, i.e., (1) stimulating the gallbladder to contract and release stored bile acids into the intestine; (2) enhancing the secretion of pancreatic digestive enzymes. Bile acids play an important role in the emulsification of fats and increasing the surface area of fats, activating pancreatic lipase and accelerating the formation of mixed micelles. In the liver, bile

acids are covalently conjugated with taurine and glycine in mammals but only with taurine in fish (Kim et al. 2007). However, recent evidence suggests that bile acids are conjugated mainly with taurine and, to a lesser extent, with glycine in the liver of fish (El-Sayed 2014). The functions of taurine in lipid digestion and the formation of bile salts have been well reviewed by Salze et al. (2015) and El-Sayed (2014). The green liver syndrome in some fish species may be caused by failed conjugation of bilirubin and biliverdin in response to a dietary deficiency of taurine (Takeuchi 2014).

Fatty acids undergo β-oxidation in various tissues to produce ATP. This process involves the conversion of long-chain fatty acyl-CoA (LCFA) to acetyl-CoA in mitochondria (Fig I-4). Mitochondrial carnitine palmitoyl transferase (CPT1), which resides on the inner surface of the outer mitochondrial membrane and requires carnitine as an essential cofactor, is a major site for the regulation of mitochondrial LCFA transport (Wu 2018). Carnitine is derived from diets and synthesized from lysine, methionine and serine. The stimulatory effect of carnitine supplementation on β-oxidation of fatty acids has been reported for many species, such as African catfish (Ozorio et al. 2010) and common carp (*Cyprinus carpio*; Sabzi et al. 2017). Similar results were also observed in fish receiving dietary supplementation with lysine and methionine (Burtle et al. 1994; Liao et al. 2014; Wang et al. 2016). However, there are conflicting reports that dietary carnitine supplementation has no effect on lipid metabolism and even increases lipid deposition in fish (Dias et al. 2001; Zheng et al. 2014). Consistent with this phenotype, dietary supplementation with carnitine (331 or 3495 mg/kg diet) up-regulated mRNA levels for lipogenic genes, increased the activities of lipogenic enzymes, and reduced mRNA levels for a lipolytic gene CPT1A in yellow catfish, compared with fish without carnitine supplementation (Zheng et al. 2014). In

animals, β-oxidation is regulated at transcriptional and post-transcriptional levels. Transcriptional regulation involves PPARs, SREBP1, and PGC-1α, whereas post-transcriptional regulation depends on the phosphorylation of acetyl-CoA carboxylase (ACC) and the allosteric inhibition of CPT1 by malonyl-CoA. The latter is formed from acetyl-CoA (a metabolite of AAs and glucose) and bicarbonate by ACC. Thus, metabolic conditions that favor lipogenesis are associated with excessive intakes of dietary AAs and starch. Based on research with mammals (e.g., Wu 2018), studies are warranted to define the mechanisms responsible for the regulation of lipid metabolism by AAs in fish.

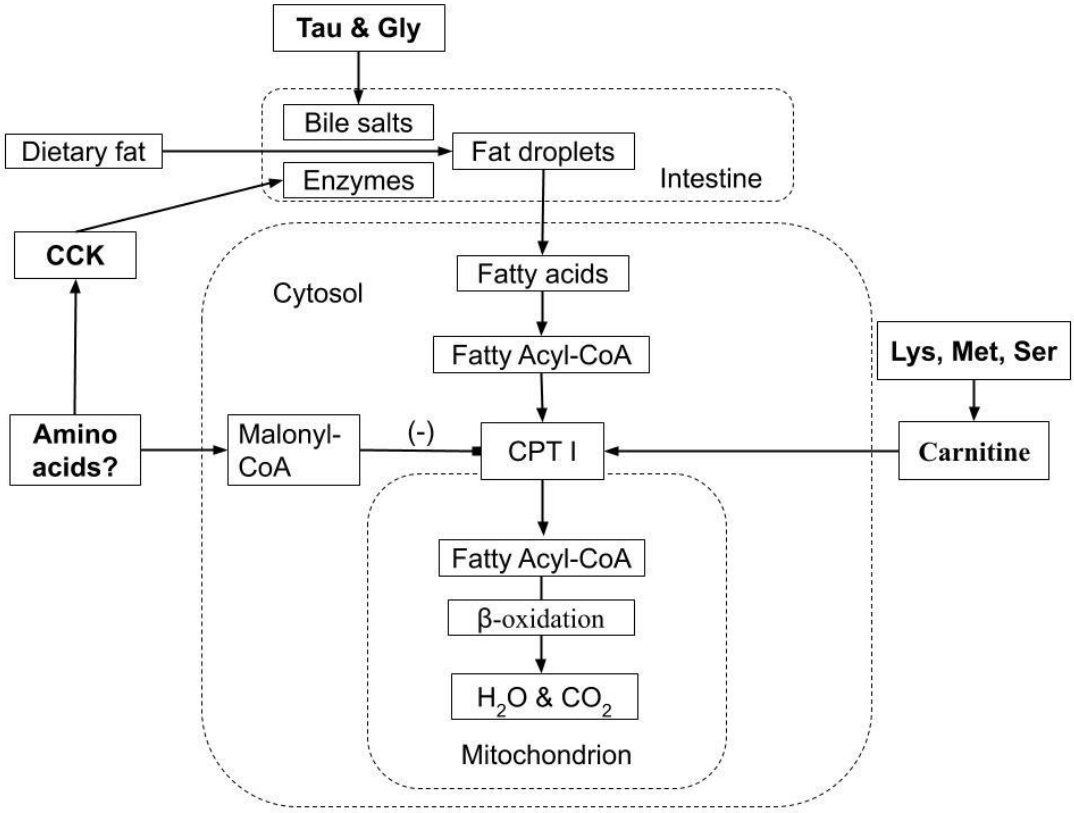


Figure I-4 Roles of AAs in the regulation of fatty acid oxidation in fish. CCK, cholecystokinin; CPT 1, Carnitine palmitoyltransferase 1; Gly, glycine; FAs, fatty acids; Lys, lysine; Met, methionine; Tau, taurine.

Nutritional methods to reduce fishmeal use in feed

Non-protein energy sources

The first method to reduce fishmeal use in feed is to reduce protein levels in diets. As a major macronutrient for fish, protein provides AAs which are essential for every type of cell in the body. AAs are also important sources of energy for intestinal tissues and immunocytes in rats, pigs and chickens (Wu 2018). Our recent study indicated that the high protein requirements of zebrafish and hybrid striped bass may be due to the high energy contribution from AAs (Jia et al. 2017). Moreover, protein synthesis requires a large amount of energy. A minimum energetic cost of protein synthesis has been estimated to be 40 mmol ATP equivalents per gram of protein synthesized, and even 50 mmol ATP equivalents per gram of protein synthesized when the use of energy for post-translational modifications and intracellular trafficking is considered (Ballantyne 2001). Thus, relationships between protein and energy intakes are critical to the efficiency with which dietary protein is partitioned into growth (Ballantyne 2001). In animals, lipids and starch can be the sources of energy, therefore potentially reducing dietary protein requirements. For example, the protein-sparing effect of dietary lipids has been well reported in many fish species, including giant croaker (*Nibea japonica*) (Li et al. 2015), starry flounder (*Platichthys stellatus*; Ding et al. 2010), grouper (*Epinephelus coioides*; Luo et al. 2005). However, this may be not a beneficial strategy for carnivorous fish species because high intake of dietary lipids and starch could result in metabolic disorders as well as decreases in food consumption and poor growth performance (Ai et al. 2004; Chaitanawisut et al. 2011; Mohanta et al. 2009; Han et al. 2014a).

Table I-1 Composition of nutrients and amino acids in feedstuffs used in experiments

	Fish meal	PBM	SBM	Rice bran	KSM	Blood meal	SPC
Dry matter (%; as-fed basis)	92.8	96.4	90.4	95.7	92.3	91.5	90.5
Crude Fat[1] (% of DM)	10.0	14.8	2.28	17.8	15.0	2.00	1.00
Crude Protein[1] (% of DM)	68.9	70.5	49.7	17.4	68.5	95.7	70.7
Amino acid (% of DM)[2]							
Ala	4.94	4.15	2.16	0.99	3.89	8.50	3.61
Arg	5.11	4.28	3.52	1.28	4.28	5.36	6.07
Cys	0.68	1.09	0.78	0.31	0.72	2.07	1.42
His	1.64	1.35	1.27	0.49	1.75	6.04	2.18
Gly	4.74	7.07	2.56	0.92	2.93	4.17	3.76
Hyp	1.67	2.35	0.08	0.01	0.14	0.56	0.14
Ile	2.99	2.44	2.25	0.56	3.69	2.75	3.92
Leu	5.09	4.51	3.81	1.18	5.47	12.3	6.06
Phe	2.76	2.42	2.45	0.75	3.38	6.32	4.21
Pro	4.10	5.23	2.74	0.79	2.49	6.85	3.62
Ser	3.10	2.77	2.35	0.80	2.84	4.90	4.52
Trp	0.76	0.67	0.70	0.21	0.76	1.43	1.26
Thr	2.93	2.69	1.95	0.64	2.91	4.31	3.42
Tyr	2.28	1.91	1.84	0.52	3.46	3.12	3.01
Val	3.48	3.02	2.31	0.87	3.65	8.92	4.02
Asp	4.19	4.01	3.46	0.74	4.48	6.73	3.53
Asn	2.84	2.67	2.34	0.70	3.12	5.06	2.29
Glu	6.50	4.93	4.68	1.22	6.07	6.94	8.29
Gln	4.38	3.55	4.14	1.16	4.03	4.68	6.81
Lys	5.33	3.68	3.10	0.76	5.58	8.96	5.30
Met	2.20	1.42	0.67	0.34	2.10	1.27	1.20
Taurine	1.01	0.50	0.00	0.00	1.74	0.17	0.00
Macro-minerals (% of DM)[1]							
Ca	5.61	3.81	0.65	0.05	2.87	0.07	0.39
P	3.75	2.55	0.69	2.24	1.49	0.20	0.68
Mg	0.26	0.17	0.37	0.99	0.68	0.02	0.36
K	1.37	0.92	2.49	1.82	0.52	0.31	2.16
S	1.27	0.74	0.42	0.21	0.84	0.50	0.52
Na	1.09	0.58	0.02	0.01	1.08	0.18	0.01
Micro-minerals (mg/kg of DM)[1]							
Zn	100	122	58	61	56	22	25
Fe	1090	296	108	106	89	2500	113
Mn	43	22	37	133	5	<1	45
Cu	7	51	16	9	79	4	9

[1] Analyzed by the Servi-Tech laboratories (Amarillo, TX, USA). [2] Amino acids in feedstuffs were analyzed as described by Li and Wu (2020).

Blood meal: chicken blood meal from Tyson Foods, AR, USA; Fishmeal: Omega Fish Meal from Corporate Headquarters of Omega Protein, Houston, Texas; KSM: Krill powder from Brine Shrimp Direct, UT, USA; PBM: Poultry by-product meal from Tyson Foods, AR, USA; SBM: 48% protein soybean meal from Hendricks Feed & Seed, IA, USA; SPC: PROFINE® Soy Protein Concentrate from Dupont, WI, USA.

Alternative sources of protein feedstuffs

Another way to reduce fishmeal use in feed is to identify alternative protein sources (NRC, 2011). The alternative protein sources (mainly plant protein sources) are less expensive than fishmeal. However, plant-protein based diets (providing the same amounts of protein as fishmeal-based diets) are often associated with reduced feed intake, reduced growth performance, and intestinal damage. Plant protein ingredients have some characteristics, such as high carbohydrate content, deficiencies in certain AAs (e.g. methionine, lysine, and tryptophan, threonine and glycine, Table I-1), low palatability, and the presence of anti-nutritional factors that limit their utilization by carnivorous fish diets (Moutinho et al. 2017). Li et al. (2016) suggested that lysine plays a significant role in protecting the intestine of fish *in vivo* and *in vitro* through the induction of expression of key antioxidant genes. Similarly, Rimoldi et al. (2016) reported that taurine supplementation to soybean meal-based diets could increase the length of villi folds, reduce the number of vacuoles, and increase the number of goblet cells. Decreases in the length of villi and the number of goblet cells were observed in turbot (*Scophthalmus maximus* L.) fed methionine-deficient diets (Gao et al. 2019). The limited level of dietary methionine in plant-protein based diets has negative effects on the immune system by impairing both cellular and humoral immune responses (Rubin et al. 2007). As noted previously, methionine is involved in polyamine and GSH syntheses, which may also affect the proliferation of lymphocytes and inflammatory processes in cells (Grimble and Grimble 1998). The positive function of dietary methionine on the immune response has been well studied in several fish species (NRC, 2011). Some studies also reported that other certain AAs, such as taurine and lysine, might modulate immune responses in aquatic animals.

Compared to plant ingredients, animal byproduct meals, such as poultry byproduct meal (PBM), krill meal, and blood meal offer several advantages, including high protein content, well-balanced AA profiles, good sources of highly digestible minerals, and the lack of known anti-nutritional factors (Table I-1). Previous studies have shown that the PBM could not replace more than 50% of fishmeal in diets for aquatic organisms (Gallagher and Degani, 1988; Fowler, 1991; Steffens, 1994). Interestingly, there are reports that the replacement of fishmeal by the PBM has reached 100% in diets for some fish species, without causing significant differences in performance parameters (Yang et al. 2006; Parés-Sierra et al. 2014). However, compared with fishmeal, these animal byproduct meals still lack some AAs, such as methionine and taurine. The use of these limiting crystalline AAs to balance AAs in diets containing alternative protein ingredients is a good strategy to enhance the growth performance of fish.

Largemouth bass

The largemouth bass (LMB, *Micropterus salmoides*) is native to North America. Because of its popularity as a sport fish and its high market value as a food, the intensive culture of LMB in the United States began in the 1960s (Brecka et al. 1996), and this fish is now reared in many other countries (including China) worldwide (Bai and Li 2018; Tidwellet al. 2019). This species has several desirable characteristics for aquaculture, such as easy adaptation to freshwater, tolerance to a wide range of temperatures, good flesh quality, and strong disease resistance. LMB is a typical carnivorous fish (eating fish and invertebrates) with a short gastrointestinal segment, and is traditionally fed directly low-value fish on farms. However, low-value fish feeding in fish cultivation will increase risks for infectious diseases and environmental pollution. As a carnivorous fish, dietary protein requirement of LMB is more than 40% (Table I-2), but an optimal level has

not been well defined. Moreover, almost 50-70% of dietary protein will be provided by fishmeal. Obviously, the expansion of aquaculture for LMB production cannot be supported only by trash fish or high inclusion levels of fishmeal in diets. To address this critical issue, the aim of this dissertation research is to determine the relative importance of AAs, fatty acids and glucose as metabolic fuels for LMB, and to reduce the use of fishmeal in its diet.

Table I-2 Reported requirements of largemouth bass (LMB) for dietary protein and lipids

Body weight of LMB (g)	Study period (days)	Crude protein requirement (% of dry diet)	Lipid requirement (% of dry diet)	References
1.8 – 6.7	36 – 50	40 – 41	10	Anderson et al. (1981)
5 – 15	26 – 68	40 – 41	10	Anderson et al. (1981)
14 – 23	64	43.6	10	Portz et al. (2001)
122 – 436	365	47	3.7	Tidwell et al. (1996)
16 – 79	84	43.4	7.6 – 17.4	Bright et al. (2005)
10 – 87	88	46 – 49	11.5 – 14	Chen et al. (2012)
8.7 – 52	56	55 – 58	13.6	Huang et al. (2017)
10 – 36	56	51.6	12.2	Cai et al. (2020)
100 – 320	56	50.5	12.2	Cai et al. (2020)
200 – 526	84	47.8	12.2	Cai et al. (2020)
13 – 42	56	47.3	10	Zhou et al. (2020)

Objective and Significance

The objectives of this dissertation research are to: (1) determine the oxidation of AAs, fatty acids, and glucose as metabolic fuels in major tissues of LMB; and (2) determine the appropriate dietary levels of protein, lipids and starch for LMB; and (3) develop low-cost diets for the fish by replacing fishmeal with plant and animal byproduct meal. Results from this work will advance our basic knowledge of the metabolism of nutrients, particularly AAs, in fish. Furthermore, the findings will help to understand why LMB as a fish species have high requirements for dietary protein and why this fish does not tolerate high levels of dietary starch. Finally, successful replacement of fishmeal with animal-source byproducts is expected to reduce the burden of fishmeal supply and promote the sustainability of global aquaculture.

CHAPTER II

OXIDATION OF ENERGY SUBSTRATES IN TISSUES OF LMB (*MICROPTERUS SALMOIDES*)

Abstract This study tested the hypothesis that AAs are oxidized at higher rates than glucose and palmitate for ATP production in tissues of largemouth bass (LMB, a carnivorous fish). Slices (10-50 mg) of liver, proximal intestine, kidney, and skeletal muscle isolated from LMB were incubated at 26 °C for 2 h in oxygenated Krebs–Henseleit bicarbonate buffer (pH 7.4, with 5 mM glucose) containing either [U-^{14}C]glucose, 2 mM alanine plus [U-^{14}C]alanine, 2 mM aspartate plus [U-^{14}C]aspartate, 2 mM glutamate plus [U-^{14}C]glutamate, 2 mM glutamine plus [U-^{14}C]glutamine, 2 mM leucine plus [U-^{14}C]leucine, or 2 mM palmitate plus [U-^{14}C]palmitate. In parallel experiments, tissues were incubated with a [U-^{14}C]-labeled tracer and a mixture of unlabeled substrates [alanine, aspartate, glutamate, glutamine, leucine, and palmitate (2 mM each) plus 5 mM glucose]. $^{14}CO_2$ was collected to calculate the rates of substrate oxidation. In separate experiments, O_2 consumption by each tissue was measured in the presence of individual or a mixture of substrates. The activities of key metabolic enzymes were also measured. Results indicated that the liver and skeletal muscle had a limited ability to oxidize glucose and palmitate to CO_2 for ATP production in the presence of individual or a mixture of substrates due to low activities of carnitine palmitoyltransferase-I, hexokinase and pyruvate dehydrogenase. In the presence of individual substrates, each AA was actively oxidized by all the tissues. In the presence of a mixture of substrates, glutamine and glutamate were the major metabolic fuels in the proximal intestine and kidney, as glutamine for the liver and aspartate for skeletal muscle. All the tissues

had high activities of glutaminase, glutamate dehydrogenase, and transaminases. Based on tissue weight, glutamine was the major metabolic fuel in LMB, followed by glutamate and aspartate in descending order. Glutamine plus glutamate plus aspartate generated 60-70% of ATP in LMB tissues.

Introduction

Animals need a constant supply of energy to maintain their basal metabolism (Alberts et al. 2002). In humans, livestock and poultry, glucose and fatty acids are the major metabolic fuels, with AAs providing < 20% of ATP (Wu 2018). However, the oxidation of AAs as an entity may contribute up to 70% of energy needs in the marine fish embryos and yolk-sac larvae (Rønnestad et al. 1999; Rønnestad and Fyhn, 2008). In support of this notion, in fish that require a dietary intake of 624 g protein to gain 1 kg of body weight, the retention of dietary protein in the body is only about 32% (Teles et al. 2019), indicating that most of the diet-derived AAs are degraded. We reported that glutamate and glutamine are extensively oxidized in the liver, kidney, and skeletal muscle of hybrid striped bass (a carnivore) and that the intestine of this fish also actively oxidizes glutamate and, to a much lesser extent, glutamine (i.e., 28% of the rate of glutamate oxidation) (Jia et al. 2017). For comparison, studies with pigs and rats indicate that glutamate, aspartate, and glutamine are the main energy substrates for their small intestine (Blachier et al. 2009; Hou and Wu 2018; Wu 1998). The yields of ATP from substrate oxidation (mol ATP/CO_2 produced) in fish tissues are as follows: glutamate, 4.5; glutamine, 4.5; alanine, 5; aspartate, 3.75; leucine, 5.75, palmitate, 6.625; and glucose, 5 (Jia et al. 2017).

Largemouth bass (LMB; *Micropterus salmoides*, a carnivorous fish) is a commercially important fish for freshwater aquaculture in North America and many other countries (e.g., China)

(Tidwell et al. 2019). Wild LMB eats animals, like fish, crayfish, worms, frogs, and insects that contain high protein levels (Howick and O'Brien 1983). As a result, this species should exhibit a metabolic strategy preferentially to catabolize AAs for ATP production. In aquaculture, LMB are fed compound feeds containing 45-50% protein (dry matter basis, Table I-2), with the retention of dietary protein in the body being 26-35%, depending on experimental conditions (Huang et al. 2017; Ren et al. 2018). Thus, most of the diet-derived AAs must be lost from the body, with their oxidation to CO_2 possibly being a major route. At present, there is no information about the oxidation of AAs, glucose and fatty acids in LMB tissues. This study was conducted with metabolic tracers to fill this critical knowledge of nutrient metabolism in fish.

Materials and Methods

Chemicals and medium preparation

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-[U-^{14}C]aspartate, L-[U-^{14}C]alanine, and [U-^{14}C]palmitic acid. Before use, ^{14}C-labeled glutamine, glutamate and leucine were purified according to method as described previously (Self et al. 2004; Jia et al. 2017).

Experimental animals and the collections of tissues

Juvenile LMB with a body weight of about 8 g were obtained from Larry's Fish Farm (Giddings, TX, USA) and housed in the Kleberg Center of Texas A&M University, as previously described (Jia et al. 2017). The photoperiod of the housing facility was maintained for 14 h per day, with lights being turned off between 10:00 PM and 8:00 AM. The fish were fed twice daily (at 9 AM and 4 PM) with a fishmeal-based diet containing 50% protein, 10% lipids and 10% starch to

apparent satiation, and reared in tanks (55 L) with deionized water (Jia et al. 2017). From the outlet of the fish tanks, the water flowed through mechanical and biological filters, and about 50% of water was changed every day. Air was supplied to the water through air stones connected to air pumps, the water salinity was maintained at 2-4 ppt. The LMB generally prefer low salinity of water (< 5 ppt), and have a high ability to adapt to the variable salinities of water in the wild environments (Hijuelos et al. 2016). Previous studies indicated that the LMB can regulate blood osmolality efficiently at surrounding salinities less than or equal to 4 ppt (Measor and Kelso 1990; Susanto and Peterson 1996). The salinities of water at 2-4 ppt are appropriate to maintain LMB growth and health. Air was supplied to the water through air stones connected to air pumps, the salinity of the water was maintained at 2-4 ppt. The water temperature was maintained at 25-26 °C. Water quality parameters were monitored daily and remained within acceptable limits [pH 6.5-7.5, $NH4^+$ (< 1 mg/L), nitrite (< 1 mg/L), nitrate (< 20 ppm), and dissolved O_2 (8 ppm)]. All experimental procedures were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

On the day of tissue collection (9:00 AM), fish (about 40 g /fish) were anesthetized with 140 ppm MS-222 (neutralized with $NaHCO_3$), followed by the dissection of the liver, proximal intestine (the anterior segment) intestine, kidney, and dorsal skeletal muscle for metabolic studies, measurements of oxygen consumption, and assays of enzyme activities. The numbers of fish used for each measurement are indicated in the footnotes of tables. The amount of tissue used for each experiment was based on its availability from fish. In our preliminary experiments, we found that the oxidation of substrates by each tissue was linear with the amount of tissue (10-50 mg) used in the incubation.

Determination of substrate oxidation

Metabolic studies were conducted as previously described (Jia et al., 2017). Briefly, all tissues were sliced into small pieces before incubation. The intestine was cut longitudinally and washed with phosphate-buffered saline to remove the remaining intestinal content, and then a piece of soft paper was used to dry water on the surface of the intestine. A tissue slices (20-30 mg for liver and intestine, 10-20 mg for kidney, and 40-50 mg for skeletal muscle) was incubated at 26 ℃ (the temperature of water in our tanks for rearing the LMB) for 2 h in 1 ml of oxygenated (95% O_2/ 5% CO_2) Krebs-Henseleit bicarbonate (KHB) buffer (pH 7.4) containing 5 mM D-glucose and 1 nM insulin. To study substrate oxidation, the KHB buffer contained one of the following combinations of a tracer plus a tracee: D-[U-^{14}C]glucose, 2 mM L-alanine + L-[U-^{14}C]alanine, 2 mM L-aspartate + L-[U-^{14}C]aspartate, 2 mM L-glutamate + L-[U-^{14}C]glutamate, 2 mM L-glutamine + L-[U-^{14}C]glutamine, 2 mM L-leucine + L-[U-^{14}C]leucine, or 2 mM palmitate + [U-^{14}C]palmitic acid. In parallel experiments, a tissue was incubated with a [U-^{14}C]-labeled tracer and a mixture of unlabeled substrates [alanine, aspartate, glutamate, glutamine, leucine, and palmitate (2 mM each) plus 5 mM glucose]. The specific radioactivity of each tracer in the incubation medium was about 30 dpm of ^{14}C-tracee/nmol carbon of tracee for amino acids and glucose, and 10 dpm of ^{14}C-tracee/nmol carbon of tracee for palmitate. Because of a limited amount of tissues from a juvenile fish, experiments involving the oxidation of individual ^{14}C-labeled substrates in the presence of mixed substrates were conducted with different fish than those used for the measurement of substrate oxidation in the presence of 5 mM glucose. We chose glutamate, glutamine, aspartate, and alanine in the oxidation study for the following reasons. First, they are generally the most abundant amino acids in many protein feedstuffs (e.g., fishmeal and soybean meal; Li and Wu

2020). Second, some of them (e.g., glutamate, glutamine and aspartate) are known to be important energy substrates in the small intestine of mammals (Blachier et al. 1999, 2009) and the proximal intestine of some fish such as hybrid striped bass (Jia et al. 2017; Li et al. 2020). Third, leucine represents branched-chain amino acids, which are also abundant in many protein feedstuffs for fish (Li and Wu 2020). We used palmitate for the study because it is the most abundant fatty acid in the LMB, which constitutes about 20% of the total fatty acids (Tidwell et al. 2007).

In all experiments, blanks (KHB buffer without a tissue) were run for each tracer (n = 6 per tracer). After a 2-h incubation period, 0.2 ml Soluene (a Trademark name for an alkaline solution) was injected through the rubber stopper into a microtube suspended within the tube, and 0.2 ml of 1.5 M $HClO_4$ was then injected through the stopper into the incubation medium. After an additional 1 h of incubation to trap the evolved $^{14}CO_2$, the microtube was transferred into a scintillation vial (containing 5 ml of liquid scintillation cocktail) to measure ^{14}C radioactivity using a Packard scintillation counter (Zhang et al. 2019). 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 as described previously (Jia et al. 2017). In our preliminary experiments, we found that the oxidation of each substrate by the tissue to produce CO_2 was linear during a 2-h period of incubation. In our preliminary experiments, we found that CO_2 production from each substrate by the tissues studied was linear during a 2-h period of incubation.

The rate of oxidation of a substrate in a tissue (CO_2/mg tissue per 2 h) was calculated as dpm of the $^{14}CO_2$ produced by the tissue divided by the specific radioactivity of the substrate in incubation medium (Jia et al. 2017). The rate of ATP production from a substrate was calculated

from the rate of CO_2 production by multiplying a coefficient (ATP/CO_2) according to the following equations:

Glutamate: $C_5H_9NO_4 + 4.5O_2 \rightarrow NH_3 + 5CO_2 + 3H_2O$ ($ATP/CO_2 = 4.5$; mol/mol)

Glutamine: $C_5H_{10}N_2O_3 + 4.5O_2 \rightarrow 2NH_3 + 5CO_2 + 2H_2O$ ($ATP/CO_2 = 4.5$; mol/mol)

Alanine: $C_3H_7NO_2 + 3O_2 \rightarrow NH_3 + 3CO_2 + 2H_2O$ ($ATP/CO_2 = 5$; mol/mol)

Aspartate: $C_4H_7NO_4 + 3O_2 \rightarrow NH_3 + 4CO_2 + 2H_2O$ ($ATP/CO_2 = 3.75$; mol/mol)

Leucine: $C_6H_{13}NO_2 + 7.5O_2 \rightarrow NH_3 + 6CO_2 + 5H_2O$ ($ATP/CO_2 = 5.75$; mol/mol)

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

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

The rate of ATP from a substrate by a whole tissue in LMB was calculated on the basis of the mean tissue weight for the 40 g LMB (n = 6). The mean weights of liver, kidney, intestine and skeletal muscle in the 40-g LMB were 1.87%, 0.12%, 0.62% and 45.0% of the whole body, respectively. Therefore, the calculated weight of liver, kidney, intestine and muscle in 40 g fish were 750, 48, 250, and 18,000 mg, respectively.

Determination of O_2 consumption

Closed-cell respirometry was used to measure the consumption of oxygen by tissues placed in KHB buffer (pH 7.4) containing different substrates, as described above (Heylam et al. 2014). Briefly, this system (a Strathkelvin Model 929 of 6-channel oxygen meter; Warner Instruments, Hamden, CT) consisted of 6 separate Strathkelvin electrode respirometers (chambers) and 6 separate Strathkelvin oxygen electrodes fitted with polypropylene membranes. Each chamber contained 1.2 ml of KHB buffer. Tissue slices (10-20 mg for liver, kidney and intestine; and 40 mg for skeletal muscle) were incubated at 26 ℃ for 30 min. The oxygen concentration was recorded over a 20-min period. The rate of oxygen consumption by a tissue was generated by the software of the instrument based on the change in oxygen concentration in the chamber solution between 10 and 20 min when oxygen concentration in the medium decreased linearly. The blanks of the chambers without tissue were recorded as described above, except that the KHB buffer did not contain any tissue, and were found to be zero. The rate of oxygen consumption was expressed per mg tissue.

Transport of nutrients by LMB tissues

The uptake of nutrients by LMB tissues was measured with the use of ^{14}C-labeled substrates as described for the oxidation experiments in the presence of a mixture of energy substrates, except that ^{3}H-inulin was included as an extracellular marker (Lei et al. 2012). Briefly, weighed tissue slices were incubated at 26 ℃ for 5 min in 1 ml of KHB 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 radiolabeled substrate remained

on the surface of tissue slices, as indicated by the extracellular marker (i.e., ^{3}H-inulin). Then the tissue was transferred into a 1.5-ml microtube containing 0.5 ml of 1 M NaOH for solubilization overnight. An aliquot (0.1 ml) of the solubilized tissue solution was mixed with 5 ml of 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).

Measurement of enzyme activities

The activities of key enzymes involved in the oxidation of AAs, glucose and fatty acids were measured at 25 ^{o}C by following the published methods. The activity of hexokinase was based on the reduction of $NADP^{+}$ into NADPH through a coupled reaction with glucose-6-phosphate dehydrogenase that was measured spectrophotometrically by an increase in absorbance at 340 nm (Yilmaz et al. 2004). The assay medium contained 50 mM Tris-HCl buffer (pH 8.0), 13.3 mM $MgCl_2$, 6.8 mM $NADP^{+}$, 16.5 mM ATP, 1.5 mM KCl and 0.67 M glucose based on the previous study with goldfish (dos Santos et al. 2010). The activity of lactate dehydrogenase (LDH) was measured by the spectrophotometric method described by Fotakis and Timbrell (2006). The assay medium contained 0.1 M sodium phosphate buffer (pH 7.0), 200 µM NADH, 0.75 mM pyruvate based on a previous study with cod (*Gadus morhua*; Zakhartsev et al. 2010). The activity of pyruvate kinase was determined by the spectrophotometric method as described by Tietz and Ochoa (1958) with some modifications. The assay medium contains 60 mM Tris-HCl buffer (pH 7.5), 4 mM $MgCl_2$, 75 mM KCl, 2 mM ADP, 1.4 mM phosphoenolpyruvate (PEP), 0.5 mM NADH, based on previous work with eel (Robert and Anderson, 1985). The activity of phosphofructokinase-1 (PFK) was determined by the spectrophotometric method described by Hengartner and Harris (1975). The assay medium contained 100 mM Tris–HCl buffer (pH 9.0), 1

mM ATP, 2 mM PEP, mM $MgCl_2$, 5 mM KCl, 0.26 mM NADH, 2 mM $MgSO_4$, 5 U/ml pyruvate kinase, and 5 U/ml LDH, based on a previous study with sea bream (*Sparus Aurata*; Mediavilla et al. 2008). The activity of pyruvate dehydrogenase was measured by a commercial kit (MAK183, Sigma Chemicals, St Louis, MO) using a coupled enzyme reaction that resulted in a colorimetric (450 nm) product proportional to the enzymatic activity. The activities of glutamate dehydrogenase, glutamate-pyruvate transaminase (alanine transaminase), and glutamate-oxaloacetate (OAA) transaminase (aspartate transaminase) were measured by using spectrophotometric methods that involved a decrease or increase in absorbance at 340 nm due to the degradation or generation of NADH, respectively (Bush et al. 2002; Wu et al. 2000). For glutamate dehydrogenase, the assay medium contained 80 mM potassium phosphate buffer (pH 7.6), 110 mM NH_4Cl, 7 mM α-ketoglutarate, 1.6 mM ADP, 160 μM NADH, based on the previous study with Antarctic fish (Ciardiello et al. 2000). The activities of liver- and kidney-type glutaminase were measured as described by Watford and Smith (1990) and Bush et al. (2002), based on the production of ammonia and glutamate from glutamine. The activity of branched-chain AA transaminase (BCAT) was measured as described by Self et al. (2004), based on the production of glutamate from leucine and α-ketoglutarate (α-KG). The assay medium contained 50 mM Tris-HCl buffer (pH 8.6), 0.08 mM pyridoxal phosphate, 5 mM α-KG, and 5 mM leucine under 50 mM Tris/HCl buffer (pH 8.6). The activity of carnitine palmitoyltransferase-I (CPT-I) was measured by using [^{14}C]carnitine as a substrate as described by Brown (2003). The activities of all enzymes are expressed as nmol/mg protein per min. All the enzyme assays were linear with time (20 min) and the amount of enzyme extract used in the solution (0.1 to 0.5 mg of tissue protein/ml). Concentrations of protein in tissue homogenate were determined by using the

Pierce™ BCA protein assay kit (Thermo Fisher, Rockford, Illinois). In our preliminary experiments, we found that the activity of each enzyme was linear with the time of incubation and the amount of protein used for the assay.

Statistical analysis

Values are expressed as means ± SEM. All data were analyzed by using the SPSS package (version 19.0, SPSS Inc, Chicago, IL, USA). The data were tested for homogeneity (the Levene's test) and normal distribution (the Kolmogorov–Smirnov test) before analysis (Wei et al. 2012). Data were analyzed by one-way ANOVA and the Student-Neman-Keuls multiple comparison test. *P*-values < 0.05 were taken to indicate statistical significance.

Results

Oxidation of individual substrates, ATP production and oxygen consumption by LMB tissues incubated in the presence of 5 mM glucose

Oxidation of individual substrates in tissues incubated in the presence of 5 mM glucose

Table II-1 summarizes data on the rates of CO_2 production from the oxidation of individual ^{14}C-labeled nutrients (glucose, palmitate, glutamate, glutamine, aspartate, alanine, and leucine) by LMB tissues incubated in the presence of 5 mM glucose. In the liver, proximal intestine, kidney, and skeletal muscle, the rates of CO_2 production from [U-^{14}C]glutamine oxidation were the highest ($P < 0.05$) among all the substrates studied, whereas the rates of CO_2 production from [U-^{14}C]palmitate oxidation were the lowest ($P < 0.05$) among all the substrates studied. In the liver, the rate of oxidation of [U-^{14}C]aspartate was the second highest ($P < 0.05$) among all the substrates studied, followed by glutamate, leucine, and alanine in descending order. In the proximal intestine, the rates of CO_2 production from [U-^{14}C]glutamate, aspartate or alanine (similar rates) were the

second highest ($P < 0.05$) among all the substrates studied, followed by glucose, leucine, and palmitate in descending order. In the kidney, the rate of CO_2 production from [U-^{14}C]glutamate was the second highest ($P < 0.05$) among all the substrates studied, followed by aspartate and alanine (similar rates), glucose, leucine and palmitate in descending order. In skeletal muscle, the rates of CO_2 production from [U-^{14}C]aspartate and [U-^{14}C]glutamate (similar rates) were the second highest ($P < 0.05$) among all the substrates studied, followed by leucine, alanine, glucose and palmitate in descending order. The liver and skeletal muscle had a limited ability to oxidize [U-^{14}C] glucose and [U-^{14}C] palmitate to CO_2 when the incubation medium contained 5 mM glucose.

Both the proximal intestine and the kidneys of LMB had higher ($P < 0.05$) rates of oxidizing all the substrates studied in the presence of 5 mM glucose, in comparison with the liver and skeletal muscle of the fish. The rates of oxidation of [U-^{14}C]glucose or [U-^{14}C]glutamine did not differ ($P > 0.05$) between the proximal intestine and the kidney, but the rates of oxidation of [U-^{14}C]glutamate, aspartate, alanine, leucine, and palmitate in the kidney were higher ($P < 0.05$) than those in the proximal intestine. The rates of oxidation of [U-^{14}C]glutamine did not differ ($P > 0.05$) between the liver and skeletal muscle. However, compared with skeletal muscle, the rates of oxidation of [U-^{14}C]glucose, [U-^{14}C]palmitate, [U-^{14}C]alanine, and [U-^{14}C]leucine in the liver were higher ($P < 0.05$), but the rates of oxidation of [U-^{14}C]glutamate and [U-^{14}C]aspartate were lower ($P < 0.05$), when the tissues were incubated with 5 mM glucose; similar results were obtained for the proximal intestine or kidney in comparison with skeletal muscle, except that the rates of oxidation of [U-^{14}C]glutamate in the proximal intestine and kidney were 279% and 581% higher ($P < 0.05$) than those in skeletal muscle, respectively.

Production of ATP from the oxidation of individual substrates in tissues incubated in the presence of 5 mM glucose

The rates of ATP production from individual substrates in LMB tissues incubated in the presence of 5 mM glucose are shown in Table II-3. The values were generally similar to those for the rates of substrate oxidation indicated previously. Note that the rates of ATP production from the oxidation of glutamine were the highest ($P < 0.05$) among all the substrates studied in the liver, proximal intestine, kidney, and skeletal muscle. The second most important substrates for ATP production varied among tissues: aspartate, leucine and glutamate (similar rates) in the liver, glutamate and alanine (similar rates) in the proximal intestine, glutamate in the kidney, and glutamate and aspartate (similar rates) in skeletal muscle. The third most important substrates for ATP production also varied among tissues: alanine in the liver, aspartate and glucose (similar rates) in the proximal intestine, alanine in the kidney, and leucine in skeletal muscle. Based on the rates of ATP production in the presence of glucose plus an energy substrate and the weights of the individual tissues, skeletal muscle (45% of the body weight) produced most ATP from all substrates per whole tissue in LMB, followed by the proximal intestine, liver, and kidney in descending order.

Oxygen consumption by tissues incubated in the presence or absence of 5 mM glucose plus an additional energy substrate

Data on the rates of O_2 consumption by tissues incubated in the presence or absence of 5 mM glucose, or 5 mM glucose plus an energy substrate are summarized in Table II-3. When energy substrates were not added to the KHB medium, the liver, proximal intestine, kidney, and skeletal muscle consumed various amounts of O_2 because of the presence of endogenous metabolic fuels

(e.g., AAs, fatty acids, glucose, lactate, and pyruvate). In the liver, proximal intestine, kidney, and skeletal muscle, the rates of O_2 consumption were the highest ($P < 0.05$) in the presence of glutamine among all the substrates studied, whereas the rates of O_2 consumption were the lowest ($P < 0.05$) in the presence of glucose alone or glucose plus palmitate among all the substrates studied. The second most important substrates for supporting O_2 consumption varied among tissues: leucine, glutamate, aspartate, and alanine (similar rates) in the liver; glutamate, aspartate and alanine (similar rates) in the proximal intestine; glutamate and aspartate in the kidney; and glutamate and aspartate in skeletal muscle. The third most important substrates for supporting O_2 consumption in the proximal intestine were glucose and leucine, whereas those in the kidney were alanine. Compared with the group of no added substrate, the addition of glucose to the KHB medium increased ($P < 0.05$) the rates of O_2 consumption by the proximal intestine and kidney by 47% and 52%, respectively, but had no effect ($P > 0.05$) on O_2 consumption by the liver or skeletal muscle. Of note, compared with the 5 mM glucose group, the addition of leucine to the KHB increased ($P < 0.05$) the rates of O_2 consumption by the liver by 129%, but did not affect ($P > 0.05$) O_2 consumption by the proximal intestine, kidney or skeletal muscle. In all these tissues, the rates of O_2 consumption were positively correlated ($P < 0.05$) with the rates of CO_2 production from energy substrates (Figure II-1).

Both the proximal intestine and the kidneys of LMB had higher ($P < 0.05$) rates of O_2 consumption in the presence or absence of added substrates, compared with the liver and skeletal muscle of the fish. Among all the tissues studied, skeletal muscle had the lowest rate of O_2 consumption per mg tissue weight under all the experimental conditions used. The rates of O_2 consumption did not differ ($P > 0.05$) between the proximal intestine and the kidney when

incubated in the presence of glucose alone, glutamine, alanine or leucine, in the absence of any exogenous substrate. However, the rates of O_2 consumption by the kidney were higher ($P < 0.05$) in the presence of palmitate (+54%), glutamate (+13%), and aspartate (+12%). Note that in the presence of glucose, leucine supported substantially O_2 consumption in the liver as did glutamate, aspartate, and alanine, but was unable to do so in the proximal intestine, kidney, and skeletal muscle.

Oxidation of individual substrates, ATP production and oxygen consumption by LMB tissues incubated in the presence of mixed substrates

Oxidation of individual substrates in tissues incubated in the presence of mixed substrates

Table I-4 summarizes the data on the rates of CO_2 production from the oxidation of individual ^{14}C-labeled substrates (glucose, palmitate, glutamate, glutamine, aspartate, alanine, and leucine) by LMB tissues incubated in the presence of mixed substrates. In the liver, proximal intestine, and kidney that were incubated under this experimental condition, the rates of CO_2 production from [U-^{14}C]glutamine oxidation were the highest ($P < 0.05$) among all the substrates studied, although the rates of CO_2 production from [U-^{14}C]glutamate oxidation in both the proximal intestine and the kidney were similar to those for [U-^{14}C]glutamine oxidation. In skeletal muscle, the rates of CO_2 production from [U-^{14}C]aspartate oxidation were the highest ($P < 0.05$) among all the substrates studied, followed by glutamine and glutamate (similar rates), alanine and glucose (similar rates), and leucine and palmitate (similar rates). In the liver, proximal intestine and kidney, the rates of oxidation of [U-^{14}C]aspartate were the second highest ($P < 0.05$) among all the substrates studied. The third most oxidized substrate varied among tissues: followed by glutamate in the liver, glucose in the proximal intestine, and glucose and alanine (similar rates) in the kidney.

In all these tissues, the rates of CO_2 production from [U-^{14}C]leucine oxidation were the lowest ($P < 0.05$) among all the substrates studied.

Both the proximal intestine and the kidneys of LMB had higher ($P < 0.05$) rates of oxidizing all the nutrients studied in the presence of mixed substrates, in comparison with the liver and skeletal muscle of the fish. Except for palmitate and leucine, the rates of oxidation of all other [U-^{14}C]-labeled substrates did not differ ($P > 0.05$) between the proximal intestine and the kidney; the rates of oxidation of [U-^{14}C]palmitate and [U-^{14}C]leucine in the kidney were higher ($P < 0.05$) than those in the proximal intestine. Except for aspartate and leucine, the rates of oxidation of all other [U-^{14}C]-labeled substrates were higher ($P < 0.05$) in the liver than those in skeletal muscle; the rates of oxidation of [U-^{14}C]aspartate and [U-^{14}C]leucine did not differ ($P > 0.05$) between the liver and skeletal muscle. Among the tissues studied, the liver had the lowest ($P< 0.05$) rates of oxidizing all the substrates except for leucine noted previously.

Production of ATP from the oxidation of individual substrates in tissues incubated in the presence of mixed substrates

The rates of ATP production from individual substrates in LMB tissues incubated in the presence of mixed substrates are summarized in Table II-5. The values were generally similar to those for the rates of substrate oxidation indicated previously. Note that the rates of ATP production from the oxidation of glutamine were the highest ($P < 0.05$) among all the substrates studied in the liver, proximal intestine, and kidney, whereas the rate of ATP production from the oxidation of aspartate was the highest ($P < 0.05$) among all the substrates studied in skeletal muscle. In both the proximal intestine and kidney, the rates of ATP production from glutamate oxidation were similar to those from glutamine oxidation. The second most important substrates

for ATP production varied among tissues: aspartate in the liver; aspartate and glucose in the proximal intestine; and aspartate, glucose and palmitate in the kidney; and glutamine and glutamine (similar rates) in skeletal muscle. The third most important substrates for ATP production also differed among the tissues: glutamate in the liver, and alanine in the proximal intestine and the kidney. In skeletal muscle, the oxidation of glucose, palmitate, alanine and leucine contributed to similar rates of ATP production in the presence of mixed substrates. Based on the rates of ATP production in the presence of mixed substrates and the weights of the individual tissues, skeletal muscle (45% of the body weight) produced most ATP from all substrates per whole tissue in LMB, followed by the proximal intestine, liver, and kidney in descending order. Under this experimental condition, the oxidation of all the studied AAs (glutamate, glutamine, aspartate, alanine and leucine) together contributed to 84%, 79%, 75%, and 82% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle, respectively; and the oxidation of glutamate plus glutamine plus aspartate contributed to 70%, 66%, 61%, and 65% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle, respectively. Notably, aspartate generated the highest amount of ATP among all substrates in the sum of all the four tissues (Figure II-2). For comparison, the oxidation of glucose contributed only to 7%, 15%, 12%, and 9% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle, respectively; and the oxidation of palmitate contributed only to 9%, 6%, 13%, and 9% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle, respectively.

Oxygen consumption by tissues incubated in the presence of mixed substrates

Data on the rates of O_2 consumption by tissues incubated in the presence of mixed substrates are summarized in Figure II-3. Under this experimental condition, the rates of O_2 consumption by

the proximal intestine and kidney (expressed as nmol/mg tissue per 30 min) were similar ($P > 0.05$) and were higher ($P < 0.05$) than those by the liver and skeletal muscle. The rates of O_2 consumption by the liver were 58% higher ($P < 0.05$) than those by the skeletal muscle. Thus, among all the tissues studied, skeletal muscle had the lowest ($P < 0.05$) rate of O_2 consumption per mg tissue weight. The rates of O_2 consumption were positively correlated with the rates of CO_2 production by different tissues of the 40g LMB (Figure II-3).

Transport of nutrients by tissues incubated in the presence of mixed substrates

Data on the rates of nutrient by tissues incubated in the presence of mixed substrates are summarized in Table II-6. In the liver and kidney that were incubated under this experimental condition, the rates of glutamine transport were the highest ($P < 0.05$) among all the substrates studied, followed by either glutamate and aspartate (similar rates) in the liver or glutamate and aspartate in the kidney in descending order. In the proximal intestine, the rates of transport of glucose, glutamate, glutamine, and alanine (similar rates) were the highest ($P < 0.05$) among all the substrates studied, followed by aspartate and leucine (similar rates). In skeletal muscle, the rates of transport of glutamate and glutamine (similar rates) were the highest ($P < 0.05$) among all the substrates studied, followed by glucose, with the rates of transport of aspartate, alanine and leucine (similar rates) being lower than those for glucose. In all of these tissues, the rates of transport of palmitate were the lowest ($P < 0.05$) among the substrates studied, and the rates of transport of glucose, palmitate and leucine being similar in the kidney.

The proximal intestine of LMB had the highest ($P < 0.05$) rates of transporting all the nutrients under investigation in the presence of mixed substrates among all the tissues studied, and the rates of transport of glutamate, glutamine, aspartate and leucine were similar between the proximal

intestine and skeletal muscle. The liver had the lowest ($P < 0.05$) rates of transporting all the substrates studied, and the rates of transport of glucose, alanine and leucine did not differ between the liver and skeletal muscle. The rates of transport of glucose in the liver, kidney, and skeletal muscle were lower ($P < 0.05$) than those for glutamate and glutamine, but the rates of transport of glucose in the liver, proximal intestine, and skeletal muscle were higher ($P < 0.05$) than those for aspartate.

Activities of key enzymes involved in the oxidation of amino acids, glucose, and fatty acids in LMB tissues

Table II-7 summarizes the activities of key enzymes involved in the oxidation of AAs, glucose, and fatty acids in LMB tissues. The activity of glutamate-pyruvate transaminase was the highest ($P < 0.05$) among all the measured enzymes in the liver, proximal intestine, and kidney, whereas the activity of lactate dehydrogenase was the highest in skeletal muscle. In both the liver and proximal intestine, enzymes with the second, third, and fourth highest activities were lactate dehydrogenase, glutamate dehydrogenase, and glutamate-OAA transaminase, respectively. In the kidney, enzymes with the second, third, and fourth highest activities were glutamate-OAA transaminase, kidney-type glutaminase, and glutamate dehydrogenase, respectively. In skeletal muscle, enzymes with the second, third, and fourth highest activities were phosphofructose kinase-1, pyruvate kinase, and glutamate-pyruvate transaminase, respectively. In all of the studied tissues, enzymes with the lowest activities were carnitine palmitoyltransferase-I, hexokinase, and pyruvate dehydrogenase.

The activities of enzymes varied among tissues. The kidney had the highest activities of BCAA transaminase, glutaminase, glutamate-OAA transaminase among the tissues studied. The

activities of glutamate-pyruvate transaminase in the kidney and skeletal muscle were similar ($P > 0.05$), but were about 116% to 141% higher ($P < 0.05$) than those in the liver and proximal intestine. Among all the studied tissues, skeletal muscle had relatively highest ($P < 0.05$) activities of carnitine palmitoyltransferase-I, lactate dehydrogenase, pyruvate kinase, phosphofructose kinase-1, and pyruvate dehydrogenase, but relatively lowest ($P < 0.05$) activities of BCAA transaminase, glutaminase, and glutamate dehydrogenase.

Discussion

Carbon isotopes, such as ^{14}C-labeled substrates, are useful for investigating the oxidation of nutrients (e.g., AAs, glucose and fatty acids) in tissues (Wu 2018). $^{14}CO_2$ produced from the tracer is collected to indicate the rate of oxidation of its tracee (e.g., energy substrate, such as glucose or glutamine). This method has been well applied to our recent studies of hybrid striped bass (Jia et al. 2017). Moreover, the rates of CO_2 production obtained from radioisotope experiments, along with data on the rates of oxygen consumption by tissues, provide a useful tool to assess their metabolic activities and the role of nutrients in generating ATP for sustaining cellular physiological processes. Furthermore, information about the activities of key enzymes in metabolic pathways can help to reveal a biochemical basis for differences in the oxidation of substrates or in the metabolic patterns of animal tissues. Using these vertically integrated approaches, we conducted the present study to identify major energy sources for LMB tissues.

Glucose oxidation and contribution to ATP production in LMB tissues

The oxidation of glucose requires its conversion into pyruvate via glycolysis, followed by the decarboxylation of pyruvate by pyruvate dehydrogenase to form acetyl-CoA. The latter enters the Krebs cycle for oxidation into CO_2, yielding ATP, GTP, NADH + H^+, and $FADH_2$. With the supply

of O_2, the reducing equivalents are oxidized to H_2O via the mitochondrial electron transport system, generating ATP. Thus, in tissues that contain mitochondria but no brown adipocytes, O_2 consumption by the tissues is positively correlated with its production of CO_2, as shown for the liver, proximal intestine, kidney, and skeletal muscle incubated in the presence of either glucose plus an additional energy substrate (Figure II-1) or mixed substrates (Figure II-2). In this study, we found that the rates of CO_2 production from glucose and the rates of O_2 consumption by each of the four tissues of LMB studied in the presence of glucose alone were all much lower than those for glutamate, glutamine and aspartate. This is consistent with the results of our previous study with hybrid striped bass (carnivores) and zebrafish (omnivores) (Jia et al. 2017). The low rates of glucose oxidation to CO_2 in LMB tissues help to explain why this fish does not tolerate high dietary levels of starch (Kamalam et al. 2017).

Mitochondria-containing tissues of fish have an active Krebs cycle to oxidize acetyl-CoA into CO_2 (Jürss and Bastrop 1995), including hybrid striped bass (Jia et al. 2017) and LMB (Table II-1) as assessed from the production of CO_2 from glutamate, glutamine and aspartate. Thus, the limited oxidation of glucose in LMB tissues may result from low rates of: (a) glucose uptake, glycolysis (the metabolic pathway for converting glucose into pyruvate), and/or pyruvate decarboxylation. To address this issue, we determined glucose transport, as well as the activities of key enzymes in glycolysis, lactate dehydrogenase, and PDH. Compared with glutamate and glutamine, the rates of glucose transport were low in the liver and kidney, comparable in the proximal intestine, and 21-25% lower in skeletal muscle. Except for hexokinase and PDH, all of the measured key enzymes were present at high activities in the liver, proximal intestine, kidney, and skeletal muscle. Thus, we suggest that low activities of hexokinase and PDH limit the

oxidation of glucose by the tissues of LMB, and the low rate of glucose transport in the liver and kidney was another contributing factor.

Palmitate oxidation and contribution to ATP production in LMB tissues

Fatty acids are primary metabolic fuels for the liver and skeletal muscle of terrestrial mammals (e.g., humans, pigs and rats) and poultry (Jobgen et al. 2006). Likewise, fatty acids are considered as the major source of energy in many fish species (NRC 2011). However, the oxidation of fatty acids is limited in the liver, proximal intestine, kidney, and skeletal muscle of hybrid striped bass and zebrafish (Jia et al. 2017). Similar results were obtained from the present study with LMB (Tables II-1 and 4). Low rates of oxidation of palmitate in the tissues of LMB may be attributed to both the low rates of its transport (Table II-6) and the low activities of carnitine palmitoyltransferase-I (Table II-7). This enzyme is responsible for the transport of long-chain acyl-CoA from the cytosol to mitochondria for their conversion into acetyl-CoA via the β-oxidation pathway, with the acetyl-CoA subsequently being oxidized to CO_2 via the Krebs cycle. Compared with glutamate, glutamine, aspartate, and alanine, the rates of oxidation of palmitate were much lower in all the tissues studied. Thus, not all fish species use fatty acids as a major energy source. In support of this view, the retention of dietary lipids in the body of some fish species can be more than 90% (Wang et al. 2016 and 2017), indicating that dietary fatty acids are primarily stored as triglycerides in those aquatic animals. As consumers increasingly demand fillets with less fats, the content of total lipids in the diets for LMB should be optimized to maximize the production and quality of fillet.

Oxidation of glutamate, glutamine and aspartate and their contribution to ATP production in LMB tissues

Glutamate and glutamine are interconverted in animals in a tissue-specific manner (Wu 2013b). Glutamate is synthesized from glutamine by glutamine synthetase, whereas glutamine is hydrolyzed into glutamate by phosphate-activated glutaminase. In terrestrial mammals, aspartate is a major nitrogenous metabolite of these two AAs. Of note, glutamate, glutamine and aspartate are highly abundant in both animal- and plant-source feedstuffs used for the diets of fish (Hou et al. 2019; Li and Wu 2020; Li et al. 2011). Dietary supplementation with dietary glutamate and glutamine improves the intestinal function of fish (Andersen and Espe 2016), as reported for terrestrial mammals (Blachier et al. 2009; Hou and Wu 2018). Likewise, dietary supplementation with aspartate can alleviate intestinal damage and enhance intestinal energy status in weanling piglets (Pi et al. 2014; Liu et al. 2017), and also improve the well-being of stressed meagre (*Argyrosomus regius*; Gonzalez-Silvera et al. 2018). A crucial biochemical basis for the function of glutamate, glutamine and aspartate in the mammalian small intestine is their role as major metabolic fuels to support the physiological function of the gut (Blachier et al. 1999; Wu 1998). Results of our past and current studies also demonstrated that glutamate and glutamine are major metabolic fuels for the small intestine of hybrid striped bass and zebrafish (Jia et al. 2017), as well as LMB (Tables II-2 and 5). Interestingly, under similar experimental conditions (i.e., in the presence of individual substrates), the rates of glutamate oxidation in the proximal intestine and skeletal muscle of LMB (Table II-1) were much lower than those for glutamine, whereas the opposite was observed in hybrid striped bass (Jia et al. 2017). Clearly, there is species difference in AA metabolism between the two species of carnivores. Additionally, the present work revealed

for the first time that aspartate was also extensively oxidized to generate ATP in the gut of fish, although there were reports that aspartate is oxidized in the liver of some fish [e.g., channel catfish (Campbell et al. 1983) and rainbow trout (French et al. 1982)]. Similar findings were obtained for the extensive oxidation of glutamate, glutamine and aspartate in the kidney, liver and skeletal muscle of LMB, of glutamate, glutamine and aspartate in the hepatocytes of little skate (*Raja erinacea*; Moyes et al. 1986); and of glutamine in the kidneys of the dogfish shark (King et al. 1983). This is consistent with the early reports of glutamine oxidation in the skeletal muscles of rats and chickens (Wu et al. 1991) as well as teleost (*Salvelinus namaycush*) and nonteleost fish (*Amia calva*) fish (Chamberlin et al. 1991).

The underlying biochemical basis for the high rates of AA catabolism is that the LMB tissues had relatively high rates of AA transport and high activities of glutamate dehydrogenase, glutaminase, glutamate-pyruvate transaminase, and glutamate-OAA transaminase (Table II-7). Thus, in the presence of mixed substrates, the oxidation of these glutamine, glutamate and aspartate together accounted for 70%, 66%, 61%, and 65% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle, respectively (Table II-5). Interestingly, in the presence of mixed substrates, the rate of oxidation of aspartate to CO_2 in the skeletal muscle of LMB was about 225% higher than that for glutamate or glutamine (Table II-4), indicating a hitherto unrecognized role of aspartate as the major metabolic fuel for this tissue. Thus, glutamate-OAA transaminase favors the oxidation of aspartate in the skeletal muscle of LMB. Because little is known about preferential metabolic fuels in skeletal muscle of fish (Weber et al. 2016), the findings from the present work filled this critical gap of knowledge about nutrient metabolism. Furthemore, our collective findings defined the quantitative importancxe of AAs, glucose and fatty acids as energy

sources for major specific tissues in LMB and support the notion that AAs are the main energy source in the whole body of aquatic animals (Jürss and Bastrop 1995). This helps to explain why fish generally require a high intake of dietary protein (624 g/kg weight gain) and the efficiency of its retention in the body is only about 32% (Teles et al. 2019).

Oxidation of alanine and leucine and their contribution to ATP production in LMB tissues

All of the LMB tissues studied in the present work readily transported both alanine and leucine (Table II-6). Alanine has been indicated as a major metabolic fuel in the hepatopancreas of the carp (Nagai et al. 1973) and the hepatocytes of rainbow trout (French et al. 1981). However, this suggestion is not consistent with findings from our work with LMB. Although the rate of alanine oxidation to CO_2 was substantially higher than that for glucose and palmitate in the liver, alanine was only a minor metabolic fuel for the liver, intestine, kidney and skeletal muscle of LMB in the presence of mixed (Table II-5) and individual (Table II-2) substrates, compared with glutamine, glutamine and aspartate. Unfortunately, in those earlier studies (French et al. 1981; Nagai et al. 1973), the authors did not study the oxidation of glutamate, glutamine, and aspartate, and, therefore, failed to recognize the major roles of these three AAs as metabolic fuels in fish tissues. In the presence of mixed substrates, the oxidation of alanine contributed to only 9%, 11%, 10%, and 9% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle, respectively (Table II-5). Although all the LMB tissues had a high activity of hepatic alanine transmainase, it is possible that a low concentration of α-KG (< 0.05 mM) in the cytosol and mitochondria of intact LMB tissues (our unpublished work) may not favor the transamination of alanine to pyruvate for further oxidation. This is consistent with the suggestion that alanine is not a major source of pyruvate in the hepatrocytes of rainbow trout (French et al. 1981). Thus, in

contrast to mammals (Wu 2018), alanine is not the major source of pyruvate in the hepatocytes of LMB.

Branched-chain amino acids (BCAAs) play an important role in whole-body nitrogen metabolism (e.g., syntheses of glutamate, glutamine, alanine and aspartate) in terrestrial animals under both physiological and pathological conditions (Wu 2013b). This may also be true for fish, such as hybrid striped bass (Zhou et al. 2018). The activity of BCAA transaminase, which initiates BCAA catabolism to form branched-chain α-keto acids (BCKAs) in cells, varies among tissues in terrestrial animals (Wu 2013b), in that the enzyme activity is relatively high in the kidney, small intestine and skeletal muscle, but is very low or nearly absent from the liver (Dawson et al. 1967; Wijayasinghe et al. 1983). Interestingly, the activity of BCAA transaminase in the liver of LMB was similar to that in the intestine and was 32% higher than that in the skeletal muscle of the fish (Table II-7). As reported by Hughes et al. (1984) for lake trout (*Salvelinus namaycush*), the activity of BCAA transaminase was particularly high in the kidney of LMB. BCKAs are further oxidized by the BCKA dehydrogenase complex to generate acyl-CoAs, which are subsequently converted to acetyl-CoA. The liver and kidneys of animals (including fish) have a high activity of BCKA dehydrogenase but their skeletal muscle and intestine have relatively lower enzyme activity (Wu 2013b; Zhou et al. 2018). Accordingly, in the presence of individual substrates, the rate of leucine oxidation to CO_2 in the liver of LMB was 65% higher than that in the skeletal muscle of the fish (Table II-1), indicating a relatively high capacity of the fish liver to catabolize BCAAs. The degradation of the BCAAs is likely inhibited by the presence of their products, namely, glutamate, glutamine, aspartate and alanine, as the rate of leucine oxidation in the liver of LMB was markedly suppressed in the presence of mixed substrates (Table II-4). Under this experimental condition, the

oxidation of alanine contributed to only 5%, 2%, 4%, and 8% of ATP production in the liver, proximal intestine, kidney, and skeletal muscle, respectively (Table II-5). Similar results were obtained from studies with hybrid striped bass and zebrafish (Jia et al. 2017). Thus, the oxidation of BCAAs per se is unlikely to be a major source of energy in all the three fish species. However, BCAAs can indirectly regulate whole-body ATP production by serving as substrates for the synthesis of glutamate, glutamine and aspartate in the tissues of fish, such as skeletal muscle (Zhou et al. 2018).

Nutritional implications of this research for LMB feeding

Aquaculture is a rapidly growing industry in agriculture to provide high-quality animal protein for human consumption. Optimum nutrition is critical for enhancing the efficiency of fish farming and reducing the cost of its operation, as feeds represent approximately 50% of the production expense (Craig et al. 2017). Among all nutrients, protein is the most expensive component in diets. Proteins are important for fish growth as they provide AAs that are the building blocks of tissue proteins, substrates for the generation of ATP and other substances that are essential to physiological processes, and signaling molecules for regulating metabolic pathways (Wu 2013b). Because the growth rates (including protein accretion) of fish are generally not higher than those for terrestrial animals (e.g., pigs and poultry), most of dietary AAs (including those that are not synthesized in the body) are not used for protein synthesis and must be disposed of through either oxidation into CO_2 and water or direct excretion into the surrounding environment. Like mammals and birds, fish canot oxidize all excess amino acids into CO_2. This may explain why fish ponds are highly susceptible for nitrogen pollution, leading to high risks for their poor health and deaths. Based on the results of our current work, we surmise that fish have particularly high requirements

for dietary protein to primarily satisfy their needs for glutamate, glutamine and aspartate. Therefore, we propose that addition of low-cost, feed-grade glutamate, glutamine, and aspartate to low protein diets may help to reduce the use of high-protein feedstuffs (e.g., fishmeal) for feeding fish. This mechanism-based innovative practice is expected to sustain the global aquaculture in the face of diminishing resources.

In conclusion, the liver, proximal intestine, kidney, and skeletal muscle had a limited ability to oxidize glucose and palmitate for ATP production, compared with glutamate, glutamine and aspartate, due to low activities of carnitine palmitoyltransferase-I, hexokinase and pyruvate dehydrogenase. This finding may explain, in part, why LMB fed commercial diets containing high starch levels develop hepatic disorders and rapidly accumulate excessive fats in the body. In the presence of a mixture of substrates, glutamine and glutamate were the major metabolic fuels in the proximal intestine and kidney, glutamine in the liver and aspartate in skeletal muscle. Aspartate was the second most important energy substrate for the liver, proximal intestine, and kidney, as were glutamine ad glutamate in skeletal muscle. All the tissues had high activities of glutaminase, glutamate dehydrogenase, and transaminases. Results of this work defined for the first-time major energy substrates for LMB and have important implications for designing the next generation of their diets to improve the efficiency of their growth and sustain their global production.

Table II-1 Production of CO_2 from the oxidation of individual nutrients in LMB tissues incubated in the presence of 5 mM glucose[1]

	Glucose (5 mM)	Palmitate (2 mM)	Glutamate (2 mM)	Glutamine (2 mM)	Aspartate (2 mM)	Alanine (2 mM)	Leucine (2 mM)
Liver	$0.35 \pm 0.02^{e,B}$	$0.21 \pm 0.01^{f,C}$	$1.06 \pm 0.07^{c,D}$	$3.27 \pm 0.24^{a,B}$	$1.41 \pm 0.07^{b,D}$	$0.75 \pm 0.04^{d,C}$	$0.91 \pm 0.04^{c,C}$
Proximal intestine	$4.55 \pm 0.50^{c,A}$	$1.41 \pm 0.12^{d,B}$	$7.23 \pm 0.52^{b,B}$	$19.1 \pm 1.6^{a,A}$	$6.91 \pm 0.18^{b,B}$	$6.97 \pm 0.33^{b,B}$	$1.81 \pm 0.14^{d,B}$
Kidney	$4.82 \pm 0.56^{d,A}$	$2.27 \pm 0.21^{f,A}$	$13.0 \pm 0.62^{b,A}$	$18.8 \pm 0.70^{a,A}$	$9.94 \pm 0.73^{c,A}$	$9.69 \pm 0.73^{c,A}$	$3.62 \pm 0.09^{e,A}$
Skeletal muscle	$0.19 \pm 0.01^{e,C}$	$0.15 \pm 0.01^{f,D}$	$1.91 \pm 0.09^{b,C}$	$3.89 \pm 0.29^{a,B}$	$2.24 \pm 0.16^{b,C}$	$0.39 \pm 0.03^{d,D}$	$0.55 \pm 0.03^{c,D}$

[1] Data, expressed as nmol CO_2/mg tissue per 2 h, are means ± SEM, n = 8. The basal KHB medium contained 5 mM glucose. Either D-[U-^{14}C]glucose, 2 mM palmitate + [U-^{14}C]palmitate, 2 mM glutamate + [U-^{14}C]glutamate, 2 mM glutamine + [U-^{14}C]glutamine, 2 mM aspartate + [U-^{14}C]aspartate, 2 mM alanine + [U-^{14}C]alanine, or 2 mM leucine + [U-^{14}C]leucine was added to the basal KHB medium.
[a-f]: Within a row, means not sharing the same superscript are different ($P < 0.05$).
[A-D]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table II-2 Production of ATP from the oxidation of individual nutrients in LMB tissues incubated in the presence of 5 mM glucose[1]

	Glucose (5 mM)	Palmitate (2 mM)	Glutamate (2 mM)	Glutamine (2 mM)	Aspartate (2 mM)	Alanine (2 mM)	Leucine (2 mM)
ATP production (nmol/mg tissue per 2 h)							
Liver	$1.75 \pm 0.08^{d,B}$	$1.36 \pm 0.09^{d,C}$	$4.78 \pm 0.29^{b,D}$	$14.7 \pm 1.1^{a,B}$	$5.27 \pm 0.28^{b,D}$	$3.73 \pm 0.20^{c,C}$	$5.25 \pm 0.24^{b,C}$
Proximal intestine	$22.7 \pm 2.5^{c,A}$	$9.34 \pm 0.81^{d,B}$	$32.5 \pm 2.2^{b,B}$	$85.8 \pm 7.2^{a,A}$	$25.9 \pm 0.66^{c,B}$	$34.9 \pm 1.7^{b,B}$	$10.4 \pm 0.80^{d,B}$
Kidney	$24.1 \pm 2.8^{e,A}$	$15.1 \pm 1.40^{e,A}$	$58.4 \pm 2.8^{b,A}$	$84.5 \pm 3.2^{a,A}$	$37.3 \pm 2.8^{d,A}$	$48.4 \pm 3.7^{c,A}$	$20.8 \pm 0.53^{e,A}$
Skeletal muscle	$0.94 \pm 0.06^{e,C}$	$0.99 \pm 0.07^{e,D}$	$8.58 \pm 0.41^{b,C}$	$17.5 \pm 1.3^{a,B}$	$8.40 \pm 0.61^{b,C}$	$1.93 \pm 0.13^{d,D}$	$3.16 \pm 0.18^{c,D}$
Estimated ATP production (µmol/whole tissue per 2 h)							
Liver	1.31 ± 0.06	1.02 ± 0.07	3.59 ± 0.22	11.0 ± 0.83	3.95 ± 0.21	2.80 ± 0.15	3.94 ± 0.18
Proximal intestine	5.68 ± 0.62	2.34 ± 0.20	8.13 ± 0.55	21.4 ± 1.8	6.48 ± 0.16	8.71 ± 0.41	2.60 ± 0.20
Kidney	1.16 ± 0.13	0.72 ± 0.07	2.80 ± 0.13	4.06 ± 0.15	1.79 ± 0.13	2.32 ± 0.18	1.00 ± 0.03
Skeletal muscle	16.9 ± 1.0	17.9 ± 1.2	154 ± 7.4	315 ± 24	151 ± 11	34.8 ± 2.4	56.8 ± 3.2

[1] Data are means ± SEM, n = 8. The basal KHB medium contained 5 mM glucose. Either palmitate, glutamate, glutamine, aspartate, alanine, or leucine (2 mM each) was added to the basal KHB medium.
[a-d]: Within a row, means not sharing the same superscript are different ($P < 0.05$).
[A-D]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table II-3 Consumption of O_2 by LMB tissues incubated in the presence of 5 mM glucose plus an additional energy substrate[1]

Addition to KHB incubation medium	Liver	Proximal intestine	Kidney	Skeletal muscle
None (i.e., no energy substrates)	$2.86 \pm 0.21^{b,C}$	$7.46 \pm 0.26^{a,D}$	$7.72 \pm 0.29^{a,E}$	$1.97 \pm 0.11^{c,C}$
5 mM Glucose	$2.98 \pm 0.18^{b,C}$	$11.0 \pm 0.63^{a,C}$	$11.7 \pm 0.35^{a,D}$	$2.15 \pm 0.08^{c,C}$
5 mM Glucose + 2 mM Palmitate	$2.93 \pm 0.25^{c,C}$	$7.35 \pm 0.35^{b,D}$	$11.3 \pm 0.44^{a,D}$	$1.93 \pm 0.05^{d,C}$
5 mM Glucose + 2 mM Glutamate	$6.61 \pm 0.24^{c,B}$	$14.4 \pm 0.55^{b,B}$	$16.3 \pm 0.55^{a,B}$	$3.45 \pm 0.09^{d,B}$
5 mM Glucose + 2 mM Glutamine	$7.68 \pm 0.22^{b,A}$	$20.2 \pm 1.1^{a,A}$	$21.6 \pm 0.62^{a,A}$	$4.46 \pm 0.11^{c,A}$
5 mM Glucose + 2 mM Aspartate	$6.59 \pm 0.31^{c,B}$	$14.7 \pm 0.70^{b,B}$	$16.4 \pm 0.41^{a,B}$	$3.62 \pm 0.12^{d,B}$
5 mM Glucose + 2 mM Alanine	$5.97 \pm 0.38^{b,B}$	$13.6 \pm 0.58^{a,B}$	$14.1 \pm 0.27^{a,C}$	$2.15 \pm 0.15^{c,C}$
5 mM Glucose + 2 mM Leucine	$6.81 \pm 0.27^{b,B}$	$10.5 \pm 0.38^{a,C}$	$11.4 \pm 0.72^{a,D}$	$2.28 \pm 0.09^{c,C}$

[1] Data, expressed as nmol/mg tissue per 30 min, are means ± SEM, n = 10. The consumption of O_2 by tissues was measured in the absence of any added energy substrate, or in the presence of either 5 mM glucose alone or 5 mM glucose plus an indicated energy substrate (2 mM).

[a-d]: Within a row, means not sharing the same superscript are different ($P < 0.05$).

[A-E]: Within in a column, means not sharing the same superscript are different ($P < 0.05$).

Table II-4 Production of CO_2 from the oxidation of individual nutrients in LMB tissues incubated in the presence of a mixture of energy substrates[1]

	Glucose (5 mM)	Palmitate (2 mM)	Glutamate (2 mM)	Glutamine (2 mM)	Aspartate (2 mM)	Alanine (2 mM)	Leucine (2 mM)
Liver	$0.23 \pm 0.02^{e,B}$	$0.22 \pm 0.02^{e,C}$	$0.59 \pm 0.04^{c,B}$	$1.15 \pm 0.07^{a,B}$	$0.89 \pm 0.06^{b,B}$	$0.28 \pm 0.01^{d,B}$	$0.13 \pm 0.01^{f,C}$
Proximal intestine	$2.65 \pm 0.22^{c,A}$	$0.80 \pm 0.07^{e,B}$	$4.65 \pm 0.20^{a,A}$	$4.86 \pm 0.31^{a,A}$	$3.85 \pm 0.17^{b,A}$	$1.90 \pm 0.20^{d,A}$	$0.29 \pm 0.04^{f,B}$
Kidney	$2.29 \pm 0.15^{c,A}$	$1.91 \pm 0.14^{c,A}$	$5.05 \pm 0.24^{a,A}$	$5.34 \pm 0.16^{a,A}$	$3.39 \pm 0.21^{b,A}$	$1.93 \pm 0.10^{c,A}$	$0.72 \pm 0.04^{d,A}$
Skeletal muscle	$0.15 \pm 0.01^{c,C}$	$0.11 \pm 0.01^{d,D}$	$0.24 \pm 0.01^{b,C}$	$0.25 \pm 0.02^{b,C}$	$0.81 \pm 0.03^{a,B}$	$0.15 \pm 0.01^{c,C}$	$0.11 \pm 0.004^{d,C}$

[1] Data, expressed as nmol CO_2/mg tissue per 2 h, are means ± SEM, n = 10.
The basal KHB medium contained a mixture of energy substrates (i.e., 5 mM glucose + 2 mM palmitate + 2 mM glutamate + 2 mM glutamine + 2 mM aspartate + 2 mM alanine + 2 mM leucine).
[a-f]: Within a row, means not sharing the same superscript are different ($P < 0.05$).
[A-D]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table II-5 Production of ATP from the oxidation of individual nutrients in LMB tissues incubated in the presence of a mixture of energy substrates [1]

	Glucose (5 mM)	Palmitate (2 mM)	Glutamate (2 mM)	Glutamine (2 mM)	Aspartate (2 mM)	Alanine (2 mM)	Leucine (2 mM)	Total
ATP production (nmol/mg tissue per 2 h)								
Liver	$1.13 \pm 0.08^{d,B}$	$1.47 \pm 0.16^{d,C}$	$2.64 \pm 0.16^{c,B}$	$5.15 \pm 0.30^{a,B}$	$3.35 \pm 0.21^{b,B}$	$1.38 \pm 0.06^{d,B}$	$0.77 \pm 0.07^{e,C}$	15.9 (84%)
PI	$13.2 \pm 1.1^{b,A}$	$5.31 \pm 0.49^{d,B}$	$20.9 \pm 0.87^{a,A}$	$21.8 \pm 1.4^{a,A}$	$14.4 \pm 0.64^{b,A}$	$9.52 \pm 0.95^{c,A}$	$1.72 \pm 0.22^{e,B}$	86.9 (79%)
Kidney	$11.5 \pm 0.76^{b,A}$	$12.7 \pm 1.0^{b,A}$	$22.7 \pm 1.1^{a,A}$	$24.1 \pm 0.71^{a,A}$	$12.7 \pm 0.81^{b,A}$	$9.63 \pm 0.47^{c,A}$	$4.13 \pm 0.22^{d,A}$	97.5 (75%)
SM	$0.73 \pm 0.06^{c,C}$	$0.72 \pm 0.09^{c,D}$	$1.10 \pm 0.04^{b,C}$	$1.13 \pm 0.09^{b,C}$	$3.03 \pm 0.12^{a,B}$	$0.75 \pm 0.04^{c,C}$	$0.65 \pm 0.02^{c,C}$	8.11 (82%)
Estimated ATP production (µmol/whole tissue per 2 h)								
Liver	0.84 ± 0.06	1.10 ± 0.12	1.98 ± 0.13	3.87 ± 0.23	2.51 ± 0.16	1.03 ± 0.05	0.58 ± 0.05	11.9 (84%)
PI	3.31 ± 0.28	1.33 ± 0.12	5.23 ± 0.22	5.47 ± 0.34	3.61 ± 0.16	2.38 ± 0.24	0.43 ± 0.05	21.8 (79%)
Kidney	0.55 ± 0.04	0.61 ± 0.04	1.09 ± 0.05	1.15 ± 0.03	0.61 ± 0.04	0.46 ± 0.02	0.20 ± 0.01	4.67 (75%)
SM	13.1 ± 1.1	13.0 ± 1.6	19.8 ± 0.80	20.3 ± 1.7	54.6 ± 2.2	13.4 ± 0.69	11.6 ± 0.43	146 (82%)

[1] Data are means ± SEM, n = 10. Values in the parentheses indicate the percentage contribution of all the AAs to ATP production in the tissue. The basal KHB medium contained 5 mM glucose. Either palmitate, glutamate, glutamine, aspartate, alanine, or leucine (2 mM each) was added to the basal KHB medium.
[a-d]: Within a row, means not sharinge the same superscript are different ($P < 0.05$).
[A-D]: Within a column, means not sharing the same superscript are different ($P < 0.05$).
PI = proximal intestine; SM = skeletal muscle

Table II-6 Transport of individual nutrients in LMB tissues incubated in the presence of a mixture of energy substrates[1]

	Glucose (5 mM)	Palmitate (2 mM)	Glutamate (2 mM)	Glutamine (2 mM)	Aspartate (2 mM)	Alanine (2 mM)	Leucine (2 mM)
Liver	$250 \pm 15^{c,C}$	$92 \pm 10^{e,B}$	$373 \pm 32^{b,C}$	$607 \pm 41^{a,B}$	$146 \pm 8^{d,C}$	$390 \pm 21^{b,B}$	$218 \pm 21^{c,B}$
Proximal intestine	$883 \pm 74^{a,A}$	$197 \pm 13^{c,A}$	$856 \pm 40^{a,A}$	$853 \pm 56^{a,A}$	$398 \pm 26^{b,A}$	$870 \pm 64^{a,A}$	$431 \pm 45^{b,A}$
Kidney	$202 \pm 26^{d,C}$	$192 \pm 34^{d,A}$	$497 \pm 37^{b,B}$	$787 \pm 53^{a,A}$	$231 \pm 28^{d,B}$	$373 \pm 21^{c,B}$	$214 \pm 24^{d,B}$
Skeletal muscle	$668 \pm 51^{b,B}$	$103 \pm 11^{d,B}$	$890 \pm 91^{a,A}$	$846 \pm 57^{a,A}$	$439 \pm 66^{c,A}$	$459 \pm 35^{c,B}$	$424 \pm 51^{c,A}$

[1] Data, expressed as pmol/mg tissue per 5 min, are means ± SEM, n = 6. The basal KHB medium contained a mixture of energy substrates (i.e., 5 mM glucose + 2 mM palmitate + 2 mM glutamate + 2 mM glutamine + 2 mM aspartate + 2 mM alanine + 2 mM leucine).
[a-f]: Within a row, means not sharing the same superscript are different ($P < 0.05$).
[A-D]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table II-7 Activities of enzymes in LMB tissues[1]

	Liver	Proximal intestine	Kidney	Skeletal Muscle
Carnitine palmitoyltransferase-I (n = 8)	$1.67 \pm 0.15^{b,I}$	$1.59 \pm 0.11^{b,J}$	$1.45 \pm 0.22^{b,K}$	$2.17 \pm 0.17^{a,G}$
BCAA transaminase (n = 8)	$9.81 \pm 0.54^{b,G}$	$9.41 \pm 0.71^{b,G}$	$24.6 \pm 1.2^{a,G}$	$7.46 \pm 0.39^{c,E}$
Liver-type glutaminase (n = 8)	15.1 ± 0.94^{F}	---	---	---
Kidney-type glutaminase (n = 8)	---	$15.9 \pm 1.1^{b,F}$	$62.0 \pm 4.5^{a,C}$	$5.21 \pm 0.41^{c,F}$
Glutamate dehydrogenase (n = 16)	$52.3 \pm 2.4^{a,C}$	$51.2 \pm 2.9^{a,C}$	$46.6 \pm 1.2^{a,D}$	$5.25 \pm 0.37^{b,F}$
Glutamate-pyruvate transaminase (n = 20)	$95 \pm 6.0^{b,A}$	$106 \pm 2.9^{b,A}$	$229 \pm 9^{a,A}$	$227 \pm 15^{a,C}$
Glutamate-OAA transaminase (n = 20)	$45.7 \pm 2.0^{b,D}$	$32.2 \pm 2.6^{c,D}$	$82.8 \pm 3.7^{a,B}$	$47.6 \pm 2.1^{b,D}$
Hexokinase (n = 20)	$5.45 \pm 0.15^{a,H}$	$3.81 \pm 0.16^{c,H}$	$5.52 \pm 0.24^{a,I}$	$4.49 \pm 0.16^{b,F}$
Pyruvate kinase (n = 20)	$38.6 \pm 1.8^{b,E}$	$26.7 \pm 0.92^{c,E}$	$37.9 \pm 2.0^{b,E}$	$225 \pm 12^{a,C}$
Phosphofructose kinase-1 (n = 20)	$38.7 \pm 2.0^{b,E}$	$15.0 \pm 0.48^{d,F}$	$29.3 \pm 1.2^{c,F}$	$577 \pm 38^{a,B}$
Lactate dehydrogenase (n = 20)	$81.1 \pm 3.5^{b,B}$	$61.2 \pm 2.6^{c,B}$	$16.8 \pm 1.0^{d,H}$	$8507 \pm 428^{a,A}$
Pyruvate dehydrogenase (n = 8)	$0.91 \pm 0.05^{c,J}$	$2.66 \pm 0.13^{b,I}$	$2.57 \pm 0.09^{b,J}$	$4.86 \pm 0.34^{a,F}$

[1] Data, expressed as nmol/mg protein per min, are means ± SEM, with the number of fish indicated in the parentheses.
[a-d]: Within in a row, means not sharing the same superscript are different ($P < 0.05$).
[A-K]: Within in a column, means not sharing the same superscript are different ($P < 0.05$).
BCAA = branched-chain AA; OAA = oxaloacetate

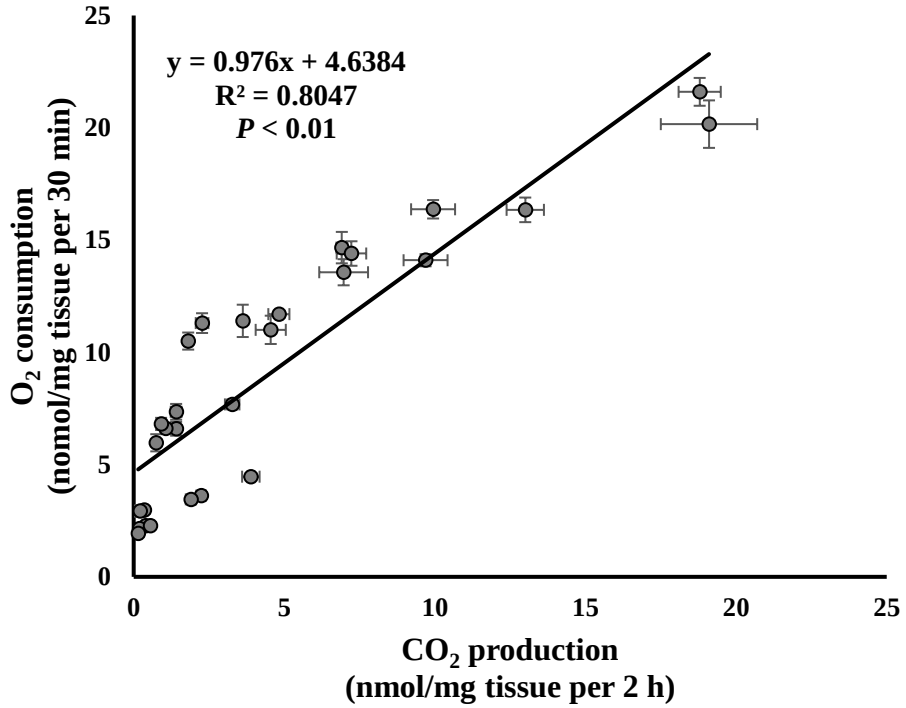
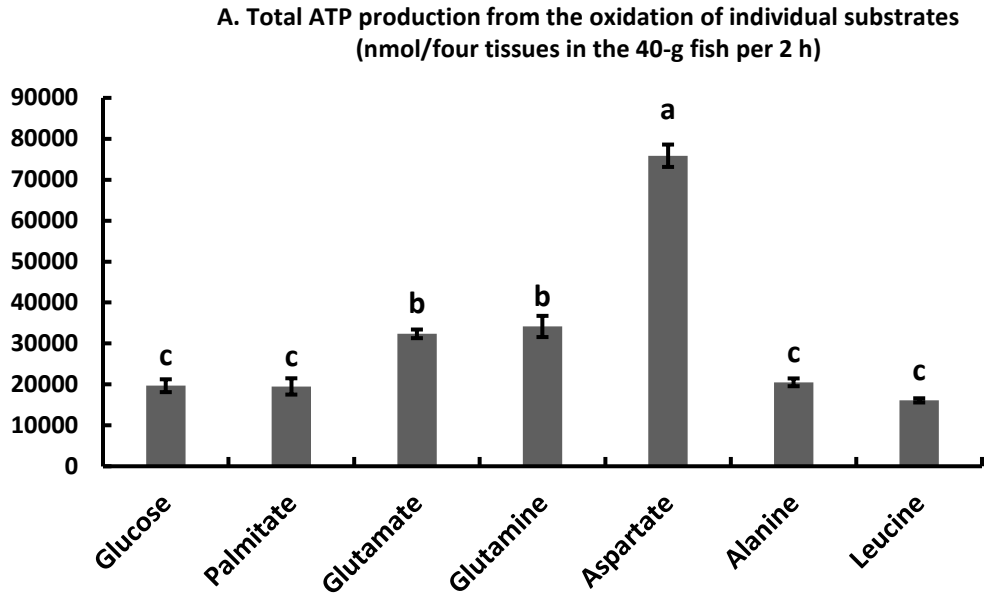


Figure II-1 The rates of O_2 consumption by tissues of largemouth bath were positively correlated with the rates of CO_2 production from the oxidation of substrates by the tissues incubated in oxygenated (95% O2/5% CO2) Krebs-Henseleit bicarbonate buffer (KHB, pH 7.4) containing 5 mM glucose plus an energy substrate. See Tables 1 and 3 for details.



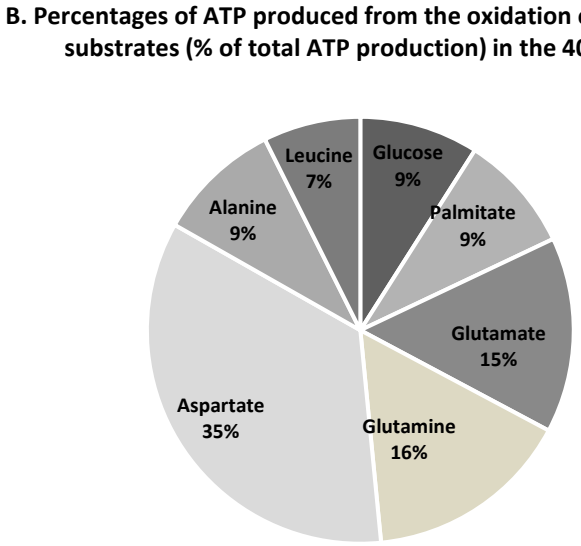
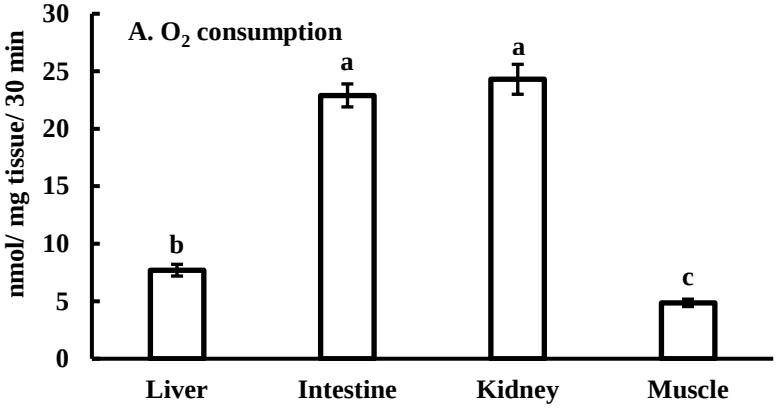
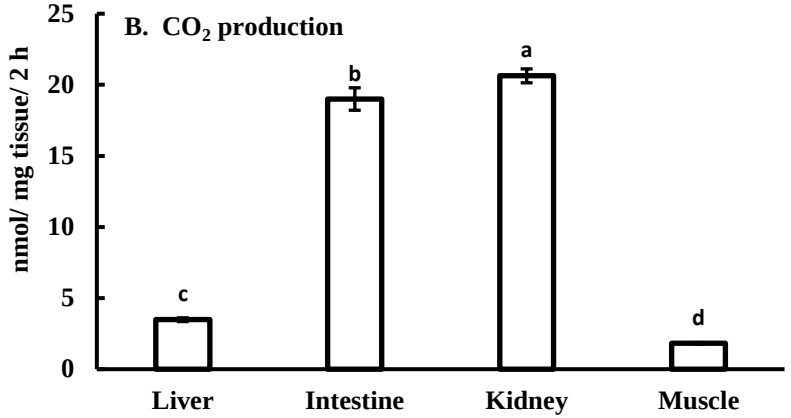


Figure II-2 Panel A. Total ATP producted from the oxidation of individual substrates (based on CO_2 production in the presence of a mixture of energy substrates) by a combination of all the studied tissues (liver, intestine, kidney and skeletal muscle) as an entity in the 40 g LMB; Paneal B. Precentages of ATP production from the oxidation of individual substrates by a combination of all the studied tissues (liver, intestine, kidney and muscle) as an entity in the 40-g LMB. a-c: Means not sharing the same superscript letter are different ($P < 0.05$).





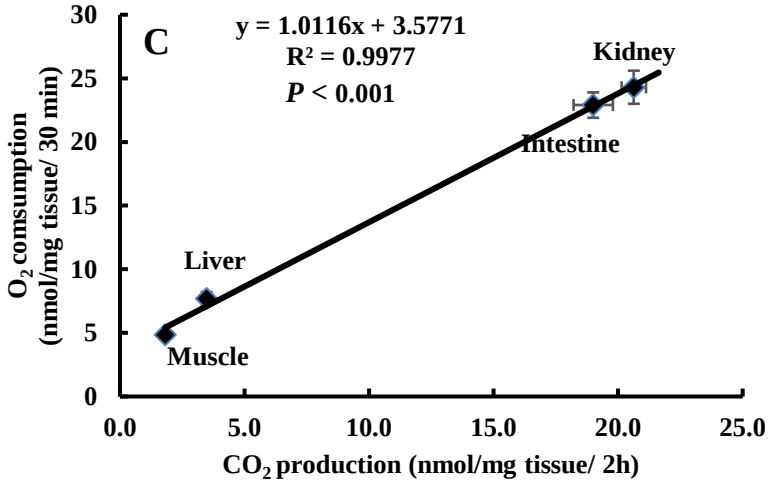


Figure II-3 Panel A. O_2 consumption by different tissues (liver, proximal intestine, kidney and skeletal muscle) in the 40-g LMB that were incubated in the presence of mixed substrates; Panel B. CO_2 production from a combination of all the studied substrates by different tissues (liver, proximal intestine, kidney and skeletal muscle) in the 40-g LMB; Paneal C. Rates of O_2 consumption were positively correlated with rates of CO_2 production by different tissues of the 40-g LMB.
Data are mean ± SEM (n = 10). a-c: Means not sharing the same superscript letter are different ($P < 0.05$).

CHAPTER III

EFFECTS OF DIETARY PROTEIN INTAKE ON THE OXIDATION OF GLUTAMATE, GLUTAMINE, GLUCOSE AND PALMITATE IN TISSUES OF LMB *(MICROPTERUS SALMOIDES)*

Abstract Largemouth bass (*Micropterus salmoides*, a carnivorous fish) prefers to utilize amino acids as energy sources rather than glucose and fatty acids. This study was conducted to determine whether the oxidation of glutamate, glutamine, glucose and palmitate in tissues might be influenced by dietary protein intake. Juvenile largemouth bass (initial weight, 18 g) were fed three iso-energetic diets containing 40%, 45% and 50% protein for 8 weeks. The growth performance, energy retention, and lipid retention of juvenile fish increased with increasing dietary protein levels. The rate of oxidation of glutamate by the intestine was much greater than that of glutamine, explaining why increasing the dietary protein content from 40% to 50% had no effect on the serum concentration of glutamate but increased that of glutamine in the fish. The liver of fish fed the 50% protein diet had a higher ($P < 0.05$) rate of glutamine oxidation than that in the other dietary group. In contrast, the kidney and proximal intestine of fish fed the 40% protein diets had higher ($P < 0.05$) rates of glutamine and glutamate oxidation in comparison with the higher protein groups, possibly to compensate for the metabolic ATP needs of. Furthermore, the rate of glucose oxidation in liver, kidney, and intestine of largemouth bass were decreased in response to increased protein intake. However, the rate of glucose oxidation in skeletal muscle was not affected by the diets. Collectively, these results indicate that the largemouth bass has an ability to regulate substrate metabolism in tissues in response to different protein intakes. Moreover, the results of growth

indicate that this fish do not make full use of high levels of starch (e.g., 22.3%) as a significant energy source.

Introduction

Protein and AAs are essential components and important energy sources for animal cells (Wu 2013 a,b). Requirements of some fish species (e.g., LMB and grouper) for dietary protein can be ≥ 45% (Shapawi et al. 2014; Luo et al. 2004), which are two- to three times those for land animals. This may be related to the use of AAs as substrates for ATP production in those fish. For example, glutamate and glutamine are the primary metabolic fuels in the small intestine, liver, kidney, and skeletal muscle of hybrid striped bass and zebrafish (Jia et al. 2017). Although protein or AAs are used very efficiently as energy sources in fish, their inclusion levels in diets should be kept to a minimum for both economic and environmental sustainability reasons. Catabolism of AAs would yield nitrogen wastes (e.g., ammonia, urea, nitrite and nitrate), possibly leading to pollution in aquatic ecosystems. As a result, optimizing utilization of AAs is fundamental for manufacturing environmentally-friendly aquafeeds and reducing feed costs (Cheng et al. 2016).

LMB is a carnivorous fish species fed by live/frozen fish or high-protein feeds on farms. This fish has a requirement for dietary protein as much as 50% (Table I-1), but with a limited ability to utilize high dietary starch (Amoah et al. 2008). The high protein requirement of LMB has been partly explained by our recent findings that AAs (such as aspartate, glutamine, and glutamate) contribute much more energy to tissues than glucose and fatty acids. Generally, an enhancement in both glycolysis and the catabolism of AAs indicates that an animal adapts well to high starch and protein intake, respectively. However, the regulation of substrate oxidation by tissues of fish in response to different nutrient intake remains largely unknown. This limits our means to

formulate fish diets with appropriate starch, AA and lipid levels that are cost-effective and also maintain animal health (intestinal and liver health). Thus, the present study was conducted to determine the effects of different dietary protein levels on growth performance, nutrient oxidation, and utilization by LMB.

Materials and Methods

Diets and Animals

Three isoenergetic experimental diets were formulated to contain 40%, 45% and 50% crude protein (CP). The ingredients and composition of diets are shown in Table III-1. Dietary protein sources were fish meal and soybean protein concentrate. Because the content of CP (% N x 6.25) in fish meal or soy protein concentrate is generally the same as that of true protein (Li et al. 2011; Li and Wu 2020), the content of true protein in these three diets was also 40%, 45%, and 50%, respectively. The levels of crude lipids in diets were 10% and were derived from fish oil, poultry fat, and soybean oil. All ingredients were thoroughly mixed using a mixer, followed by the addition of oil and water to form a moist dough. The diets were produced as pellets by using a screw extruder and then dried feeds until dry matter content was 97.0% at 50℃. The pellets were then placed in plastic bags and stored at -20℃ until use within 10 weeks after preparation.

Juvenile LMB were obtained from a commercial fish farm (Larry's Fish Farm, Giddings, TX, USA) and housed in the Kleberg Center of Texas A&M University, as previously described (Jia et al. 2017). The photoperiod of the housing facility was maintained for 14 h light per day, with lights off between 10:00 PM and 8:00 AM. Prior to starting the experiments the fish were acclimated to their tanks and fed a commercial diet for 2 weeks. There were 4 tanks (55 L of water/tank) for each dietary treatment. At the beginning of this trial, 12 fish with a similar size (a

mean initial body weight of 18.3 ± 0.1 g per fish) were randomly distributed into each tank. Air was supplied through air stones connected to air pumps, salinity was maintained at 2-4 ppt by synthetic sea salt (Instant Ocean, VA). The quality parameters of water (pH, ammonia, nitrite, and nitrate) were monitored daily and were within acceptable limits. Fish were hand-fed experimental diets to apparent satiation at 09:00, 15:00 and 20:00. Total feed consumption was recorded every day. About 50% water in tank was replaced every day. The feeding trial lasted for 56 days.

Sample collection

At the beginning of the feeding trial, 30 fish were randomly selected from the pool of fish, euthanized with 140 ppm MS-222 (neutralized by an appropriate amount of $NaHCO_3$), and frozen at -80 ^oC for subsequent analysis of whole-body composition. At the termination of the feeding trial, all fish were food-deprived for 24h. Then, total weights were recorded for each tank. Four fish per tank were randomly selected for the analysis of whole-body composition. On the last day of the trial, blood was collected with a hypodermic syringe from the caudal vein at various time points after feeding. Blood samples were centrifuged at 8000 g for 2 min. The supernatant fluid (serum) was obtained and stored at −80 ℃ until analysis.

Chemical analyses and hematology assays

Crude protein (N × 6.25) was determined by the Kieldahl method in Servi-Tech laboratories (Amarillo, TX, USA). Moisture was determined by drying in an oven at 105 ^oC to a constant weight. Lipids were extracted from the samples by using chloroform/methanol (2:1 v/v) according to the method of Folch et al. (1957). Ash was determined using a muffle furnace at 550 ℃ for 12 h. Gross energy content was calculated based on 22.6 kJ/g, 39.3 kJ/g and 17.2 kJ/g for protein, lipids, and glycogen/starch, respectively (Wu 2018).

Glucose in serum was determined using a fluorometric method involving hexokinase and glucose-6-phosphate dehydrogenase (Fu et al. 2005). Ammonia and lactate in serum was determined using a fluorometric method involving glutamate dehydrogenase and lactate dehydrogenase, respectively. AAs in serum were measured using HPLC methods (Collins et al. 2007). Briefly, the plasma sample (0.1 mL) was deproteinized with 0.1 mL of 1.5 mM $HClO_4$, followed by the addition of 0.05 mL of 2 mM K_2CO_3. The mixture was centrifuged at 10,000 g for 1 min, and 0.1 mL of the supernatant fluid was analyzed by using HPLC (Waters, Milford, MA, USA).

Determination of substrate oxidation

Oxidation of substrates was determined by using ^{14}C-labeled tracers as previously described (Chapter II). Fish were anesthetized with 140 ppm MS-222 (neutralized by an appropriate amount of $NaHCO_3$) before the liver, proximal intestine, kidney, and dorsal skeletal 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 were used to dry the water on the surface of the intestine. All tissues were sliced into small pieces before incubation. The incubation medium was the Krebs-Henseleit bicarbonate (KHB) buffer (pH 7.4) containing 1 nM bovine insulin, a mixture of energy substrates (5 mM D-glucose, 2 mM L-glutamate, 2 mM L-glutamine, 2 mM L-leucine, 2 mM palmitate, 2 mM L-alanine, and 2 mM L-aspartate), and one of the following tracers: D-[U-^{14}C]glucose, L-[U-^{14}C]glutamine, L-[U-^{14}C]glutamate, L-[U-^{14}C]leucine, L-[U-^{14}C]aspartate, L-[U-^{14}C]alanine, and [U-^{14}C]palmitic acid (250 dpm/nmol). A weighed tissue slice (10-30 mg for per liver, kidney and intestine; 40-50 mg for skeletal muscle) was incubated at 26 ℃ for 2 h in 1 ml of oxygenated (95% O_2 /5% CO_2) KHB buffer. Incubation

media with each tracer but no tissue was run as blanks (n = 4). After a 2-h period of incubation, the reaction was terminated by injecting 0.2 ml 1.5 M $HClO_4$, through the tube stopper, into the incubation medium, followed by adding 0.2 ml Soluene, through the tube stopper, into a microtube suspended within the tube to collect $^{14}CO_2$. ^{14}C radioactivity was measured by using a Packard scintillation counter (Zhang et al. 2019). The liquid scintillation cocktail used 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 in 500 ml toluene and 500 ml of 2-methoxyethanol (Wu 21997).

Calculations and statistical analysis

The rate of oxidation of a substrate by a tissue (CO_2/mg tissue per 2h) was calculated as dpm of the $^{14}CO_2$ produced by the tissue divided by the specific radioactivity of the tracer in incubation medium (Jia et al. 2017). The rate of ATP production from a substrate was calculated from the rate of CO_2 production by multiplying the coefficient (ATP/CO_2), as described in Chapter II.

Results are expressed as means ± SEM. All data on body weight and metabolic profiles were analyzed by using one-way ANOVA and the Student Newman Keuls multiple comparison test. For post-feeding concentrations of metabolites, two-way ANOVA (CP × time) was used to analyze the main effects (time and protein) and their interactions. They were tested for homogeneity (the Levene's test) and normal distribution (the Kolmogorov–Smirnov test) before analysis. Data on feed conversion ratio (FCR; feed intake/weight gain; g/g) were analyzed by regression analysis. All analyses were performed by using the SPSS package (version 19.0, SPSS Inc, Chicago, IL, USA). Probability values ≤ 0.05 were taken to indicate statistical significance.

Results

Growth and feed utilization

Data on the growth performance and feed utilization of LMB are shown in Table III-7. Daily feed intakes (DFI) of fish did not differ among the dietary groups ($P > 0.05$). The body weight gains of LMB, as well as the retention (or productive value, %) of lipids and energy in the body increased with increasing the dietary CP level from 40% to 50% ($P < 0.05$). Fish fed the 50% CP diet had a higher rate of growth than those fed the 40% CP diet ($P < 0.05$). The FCR value decreased linearly ($R^2 = 0.37$) with increasing the dietary protein level from 40% to 50% ($P < 0.05$). The retention of dietary nitrogen in the body was not affected by different diets ($P > 0.05$).

Concentrations of metabolites in serum

The post-feeding concentrations of AAs are presented in Tables III- 8 and 9. Except for arginine, the concentrations of AAs, ammonia, glucose and lactate in serum increased after feeding ($P < 0.05$). Increasing the dietary CP level from 40% to 50% increased ($P < 0.05$) the concentrations of glutamine, taurine, ornithine, threonine, valine, isoleucine, leucine and lysine in the serum of LMB, but did not affect those of arginine, tryptophan, methionine, aspartate, glutamate, asparagine, serine, glycine, citrulline, alanine, tyrosine, ammonia and lactate. In contrast, the concentrations of glucose were lower ($P < 0.05$) in the serum of LMB fed the 50% protein diet than in fish fed the 40% protein diet.

Oxidation of energy substrates and ATP production

Data on the production of CO_2 and ATP from different substrates in tissues of LMB are summarized in Tables III-2 to III-5. The rates of CO_2 and ATP production from glucose in the liver were higher in the 40% CP group ($P < 0.05$) than those in the 45% and 50% CP groups. The

liver of fish fed the 50% CP diet had a higher rate of glutamine oxidation ($P < 0.05$) than those fed the 40% and 45% CP diets. The rates of CO_2 and ATP production from glutamate or palmitate in the liver were not affected by dietary protein intake ($P > 0.05$). In the kidney, the rates of CO_2 and ATP production from palmitate, glutamine, and glucose were higher in fish fed the 40% CP diet than those fed the 45% and 50% CP diets ($P < 0.05$), but the rate of oxidation of glutamate was not affected by dietary protein intake ($P > 0.05$). In the intestine, the rates of glucose, glutamine and glutamate oxidation, and the rates of ATP production from these nutrients in the 40% CP group were higher than those in the 45% and 50% CP groups ($P < 0.05$), but rate of palmitate oxidation was not affected by dietary protein intake ($P > 0.05$). In the skeletal muscle of LMB, the rates of oxidation of all the studied nutrients or the rates of ATP production from them did not differ among fish fed the 40%, 45% and 50% CP diets ($P > 0.05$).

Discussion

Dietary protein is the major source of energy for many fish species, but little is known about the regulation of oxidation of energy substrates in their tissues by dietary protein intake. This study indicated that fish fed the 50% CP diet had a higher growth rate than those fed the 40% CP diet. This is consistent with previous results that LMB require diets containing more than 45% protein for optimum growth (Anderson et al. 1981; Huang et al. 2017). Carbohydrate is not required by fish, but can be an energy source for reducing protein catabolism (NRC 2011; Wilson 1994). The protein-sparing effects of digestible carbohydrate have been reported in several fish species (Erfanullah and Jafri 1995; Wang et al. 2005). In this study, LMB fed 40%, 45% and 50% CP diets had a similar rate of retention of dietary nitrogen in the body, indicating that more protein intake from the diet resulted in more absolute amount of protein deposited in the body. This explains our

observation that the FCR (the lower the value, the higher the efficiency of dietary protein) decreased linearly with increasing the dietary CP from 40% to 50%. Although carbohydrate is a source of the energy, it is less important than dietary protein due to a low rate of glucose oxidation by fish species (Cowey and Sargent 1977; Wang et al. 2016 a,b).

Except for skeletal muscle, all the tissues studied had higher rates of glucose oxidation when fish fed the 40% CP diet, in comparison with the 45% and 50% CP diets. In vertebrates, the liver is regarded as the central organ in controlling glucose homeostasis by serving as a consumer and a producer of glucose (Kamalam et al. 2017), and the hepatic capacity on metabolizing glucose can be up-regulated in response to high dietary starch intake. For example, pyruvate kinase or glucokinase activities in the hepatopancreas increased as the dietary starch increased from 24% to 32% in gibel carp (*Carassius auratus* var. gibelio; Tan et al. 2009) and European sea bass (*Dicentrarchus labrax*; Enes et al. 2006). The activities of glucokinase, pyruvate kinase, and phosphoenolpyruvate carboxykinase (PEPCK) increased with dietary carbohydrate/lipid ratios increasing from 0.39 to 1.34 in large yellow croaker (*Larmichthys crocea*; Zhou et al. 2016). In this study, it is also very interesting to observe that the intestine and kidney could increase glucose oxidation rate in response to high levels of dietary starch when the dietary protein level was decreased. This agrees with previous results that the intestine plays an important role in glucose homeostasis in some fish species through both glycogen storage and glucose oxidation (Polakof et al. 2010; Soengas et al. 2006; Chen et al. 2017).

Skeletal muscle represents about 45% of the whole-body weight in LMB. Thus, this tissue produces the largest amount of ATP and is primarily responsible for glucose disposal. There is a suggestion that the limited utilization of glucose by peripheral tissues may be the main reason for

the persistent hyperglycemia in fish (Kamalam et al. 2017). In support of this view, we found that the skeletal muscle of LMB did not increase glucose oxidation rate. In mammals and fish, GLUT4 is the main glucose transporter for taking up glucose from arterial blood (Capilla et al. 2004; Díaz et al. 2007). Compared with mammals, GLUT4 in the muscle of fish has a lower affinity for glucose and wider substrate specificity (Marín-Juez et al. 2013), and its intracellular traffic also differs (Marín-Juez et al. 2013; Díaz et al. 2007). All of those findings may explain the known lower ability of fish to regulate glucose utilization by their skeletal muscles. Therefore, although there are recommendations that dietary carbohydrates be supplemented to feeds of fish to maximize their growth, reduce their nitrogen excretion, and minimize the use of dietary protein for energy (Wang et al. 2016b; Hemre et al. 2002), present results indicated that inclusion of 22.3% starch in the 40% CP diet did not result in higher rates of weight gains, nitrogen retention, or FCR, compared with either the 45% CP diet with 15.8% starch or the 50% CP diet with 9.2% starch. This result indicates that starch could not spare dietary protein for LMB fed the 40% CP diet. Thus, because carnivorous fish species poorly use dietary carbohydrates (Wilson 1994), excess starch or glycogen in diets usually result in fatty fish, impaired physiological function, reduced feed consumption, and reduced feed efficiency (Mohanta et al. 2009). This metabolic problem can be ameliorated by the use of protein or AAs to replace some digestible carbohydrate in diets.

Results of our recent work have shown that AAs, including glutamate and glutamine, are more important energy substrates than glucose and lipids for tissues of hybrid striped bass (Jia et al. 2017). In the present study, fish fed diets with 50% CP had a higher rate of hepatic glutamine oxidation than those fed the 40% and 45% CP diets. This is in line with the previous study that the whole-body oxidation rate of dietary glutamate was higher in rainbow trout fed a 35% protein diet

than those fed a 10% protein diet (Kim et al. 1992). The dietary composition and protein level can influence the activities of enzymes related to AA catabolism (Ballantyne 2001). For example, increases in the activities of transaminases (ALT and AST) occur in Nile tilapia fed a 45% protein diet, when compared with those fed 25% and 35% protein diets (Abdel-Tawwab et al. 2010). High ratios of protein/carbohydrate in diets also increase ALT and AST activities in the liver of *Sparus aurata* (Metón et al. 1999) and *Sparus aurata* L (Fernández et al. 2007). A high-protein diet (61% protein) increased the hepatic glutamate dehydrogenase activity in rainbow trout, compared with a 46% protein diet by 100% (Sanchez-Muros et al. 1998). In contrast to the liver, the rates of glutamine oxidation in the kidney and intestine of LMB fed the 40% CP diet were higher than those in fish fed the 45% and 50% CP diets. One possible reason is that the amount of glutamine in the 40% CP diet was insufficient and the rate of oxidation of this AA must be increased to compensate for the metabolic needs of the intestine for glutamine by the kidney and intestine. Similarly, compared with diets with adequate protein, diets with low or insufficient levels of protein result in higher rates of whole-body oxidation of AAs (likely to provide energy) and, therefore, lower rates of retention of dietary nitrogen in the body, as reported for some fish species, such as juvenile red spotted grouper (*Epinephelus akaara*; Wang et al. 2016a) and bluegill sunfish (*Lepomis macrochirus*; Yang et al. 2016). This is very different from terrestrial animals (e.g., pigs and poultry) in which the rates of oxidation of AAs are generally reduced in response to low intakes of dietary protein (Wu 2018).

An important feature of the current study is that all experimental diets for LMB contained the same levels of lipids and energy (Table III-1). As the dietary protein level increased, the dietary starch level decreased. In land animals, high protein intake can stimulate the oxidation of fatty

acids through activating mitochondrial biogenesis and PGC1α expression (Wu 2018). Thus, we expected that fish fed the 50% CP diet would have a lower rate of lipid deposition in the body. However, the opposite result was observed. Specifically, LMB fed the 50% CP diet had higher rates of retention of dietary lipids in the body than those fed the 40% CP diet (Table III-2). This unexpected result may be explained by the use of dietary AAs for whole-body lipid synthesis. AAs are the best precursors for fat production in some fish species (Nagai and Ikeda 1971, 1972). In response to high protein intake, more AAs may be available for conversion into fatty acid synthesis, thereby contributing to high rates of retention of dietary energy and lipids in the body of LMB. Because adequate content of fat in fillet is favorable to consumers, fish should be provided with sufficient protein or AAs in diets.

Endogenous and exogenous nutrients are delivered to cells in animals through blood transport (Ballantyne 2001). Previous reports suggested that concentrations of free AAs in the plasma or serum of animals (including fish) increase after feeding to peak levels before returning to their pre-feeding values (Kultz and Jurss 1993). In terrestrial monogastric animals such as pigs and poultry, concentrations of AAs in plasma generally reach plateau values at 1 to 1.5 h after feeding and fall at 2 h after feeding to the pre-feeding values within 6 h after feeding (Wu 2018). However, LMB had a very different time-dependent pattern of the appearance of dietary AAs in plasma (Tables III-8 and 9) than mammals and birds. For example, plateau levels of most AAs in the serum of the fish were maintained between 2 and 12 h after feeding (Tables III-8 and 9). This may be due to slow rates of the intestinal digestion of dietary protein and/or the intestinal absorption of AAs into the portal vein. Except for a few AAs (e.g., glutamate, glutamine and aspartate), the concentrations of most AAs in the plasma of LMB were much higher than those in land animals (e.g., swine and

poultry) (Wu 2018), reflecting a higher intake of dietary protein in the former and possibly lower rates of uptake by the skeletal muscle of the former. The observation that increasing the dietary protein content from 40% to 50% had no effect on the serum concentration of glutamate but increased that of glutamine in LMB (Table III-8) may be explained by the our present finding that the rate of oxidation of glutamate by their intestine was much greater than that of glutamine (Table III-4). The observation that the serum concentrations of both glutamate and glutamine were relatively low in LMB although they were fed diets containing high levels of both AAs (Table III-8) supports the notion that glutamate and glutamine are extensively catabolized by the intestine of the fish (Li et al. 2020). Because these two AAs are among the most abundant AAs in the body of LMB, they must be synthesized from BCAAs and other AAs in a tissue-dependent manner. Of note, the concentrations of the precursor AAs (e.g., BCAAs, phenylalanine, and threonine) of glutamate and glutamine in serum were elevated with increasing the dietary CP content from 40% to 45% (Table III-9) to support endogenous syntheses of glutamate and glutamine in the fish.

In conclusion, the results of this study indicated that LMB had a higher ability to utilize glutamate and glutamine as energy substrates than glucose and fatty acids. Rates of glucose oxidation in the liver, kidney, and intestine of the fish fed the 40% CP diet with 22.3% starch were higher than those fed the 45% and 50% CP diets with 15.8% and 9.2% starch, respectively. The rates of glutamate and glutamine oxidation in these tissues could be regulated by dietary protein intake in a tissue-specific manner to meet their metabolic needs. Most variables of substrate oxidation did not differ between fish fed the 45% and 50% CP diets. Thus, these metabolic data indicate that the optimum requirement of juvenile LMB for dietary protein is 45% (DM basis).

Table III-1 Composition and proximate analyses of experimental diets

Dry ingredient [g/100 g of dry matter (DM)]	40% Protein	45% Protein	50% Protein
Fish meal [1]	44.51	50.07	55.63
SPC[2]	11.22	12.62	14.03
Soybean oil[3]	0.60	0.58	0.57
Poultry fat [4]	3.17	3.17	3.17
Fish oil menhaden[5]	1.22	0.61	0.00
Dextrinized starch[6]	22.30	15.78	9.20
Vitamin premix[7]	1.00	1.00	1.00
Mineral premix[8]	1.00	1.00	1.00
Cellulose[9]	13.84	14.03	14.27
CMC[10]	1.00	1.00	1.00
Proximate composition			
Dry matter (%, as-fed basis)	96	96	96
Crude protein (g/100 g DM)	40	45	50
Crude fat (g/100 g DM)	10	10	10
Phosphorus (g/100 DM)	1.5	1.6	1.9
Energy (kJ/g DM)	16.7	16.7	16.7

[1] Omega Fish Meal (Corporate Headquarters of Omega Protein, Houston, Texas).
[2] PROFINE® Soy Protein Concentrate (Dupont, WI, USA).
[3] Nutrioli Pure Soybean Oil (Ragasa, N.L., Mexico).
[4] Chicken fat (Tyson Foods, Arkansas, USA).
[5] Fish oil (Paragon, Illinois, USA).
[6] Maltodextrin (Baolingbao Biology, Shangdong, China).
[7]Vitamin premix (g/kg): vitamin A, 2.31; vitamin D3, 2.02; vitamin E, 20.00; vitamin K3, 1.2; vitamin C, 30.00; vitamin B5, 10.87; inositol, 15.00; niacin, 14.00; vitamin B6, 3.04; vitamin B2, 3.00; vitamin B1, 3.26; biotin, 0.15; folic acid, 0.6; vitamin B12, 0.02; Choline chloride, 135.00; Cellulose, 894.53.
[8] Mineral premix (g/kg): NaCl, 363.88; $MgSO_4.7H_2O$, 586.67; $FeSO_4.7H_2O$, 22.22; $AlCl_3.6H_2O$, 0.67; KI, 0.67; $CuSO_4.5H_2O$, 2.22; $MnSO_4$, 4.67； $CoCl.6H_2O$, 0.86; $ZnSO_4.7H_2O$, 18.09; Na_2SeO_3, 0.06.
[9] Microcrystaline cellulose 102 (Blue Diamond Growers, California, USA).
[10] Sodium carboxy methyl cellulose (Pro Supply Outlet, California, USA).

Table III-2 Production of CO_2 and ATP from the oxidation of nutrients by the liver of LMB fed diets containing 40%, 45% or 50% protein

Diet	Palmitate	Glutamate	Glutamine	Glucose
CO_2 Production (nmol CO_2/mg tissue per 2h)				
Protein (40%)	0.22 ± 0.01	0.57 ± 0.03	0.67 ± 0.06[b]	0.42 ± 0.05[a]
Protein (45)	0.19 ± 0.01	0.53 ± 0.04	0.57 ± 0.05[b]	0.29 ± 0.02[b]
Protein (50)	0.20 ± 0.01	0.64 ± 0.03	0.88 ± 0.04[a]	0.24 ± 0.02[b]
ATP Production (nmol CO_2/mg tissue per 2h)				
Protein (40)	1.49 ± 0.09	2.56 ± 0.13	3.00 ± 0.28[b]	2.12 ± 0.25[a]
Protein (45)	1.24 ± 0.07	2.39 ± 0.17	2.58 ± 0.21[b]	1.44 ± 0.11[b]
Protein (50)	1.32 ± 0.09	2.87 ± 0.15	3.95 ± 0.16[a]	1.20 ± 0.10[b]

Data are mean ± SEM, n =13.
[a-b]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table III-3 Production of CO_2 and ATP from the oxidation of nutrients by the kidney of LMB fed diets containing 40%, 45% or 50% protein

Diet	Palmitate	Glutamate	Glutamine	Glucose
CO_2 Production (nmol CO_2/mg tissue 2h)				
Protein (40%)	2.23 ± 0.06[a]	4.78 ± 0.26	5.67 ± 0.26[a]	3.31 ± 0.18[a]
Protein (45%)	1.89 ± 0.13[b]	4.39 ± 0.25	4.53 ± 0.16[b]	2.59 ± 0.12[b]
Protein (50%)	1.86 ± 0.13[b]	4.10 ± 0.22	4.27 ± 0.17[b]	2.13 ± 0.17[b]
ATP Production (nmol ATP/mg tissue 2h)				
Protein (40%)	14.8 ± 0.4[a]	21.5 ± 1.2	25.5 ± 1.2[a]	16.6 ± 0.9[a]
Protein (45%)	12.5 ± 0.9[b]	19.8 ± 1.1	20.4 ± 0.7[b]	13.0 ± 0.6[b]
Protein (50%)	12.3 ± 0.9[b]	18.4 ± 1.0	19.2 ± 0.8[b]	10.7 ± 0.8[b]

Data are mean ± SEM, n =13.
[a-b]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table III-4 Production of CO_2 and ATP from the oxidation of nutrients by the intestine of LMB fed diets containing 40%, 45% or 50% protein

Diet	Palmitate	Glutamate	Glutamine	Glucose
CO_2 Production (nmol CO_2/mg tissue per 2 h)				
Protein (40%)	0.63 ± 0.05	4.72 ± 0.16[a]	2.96 ± 0.19[a]	2.54 ± 0.25[a]
Protein (45%)	0.62 ± 0.08	3.22 ± 0.12[b]	2.42 ± 0.22[b]	1.34 ± 0.08[b]
Protein (50%)	0.53 ± 0.05	3.52 ± 0.17[b]	2.14 ± 0.19[b]	1.64 ± 0.07[b]
ATP Production (nmol ATP/mg tissue per 2 h)				
Protein (40%)	4.18 ± 0.32	21.2 ± 0.7[a]	13.3 ± 0.9[a]	12.7 ± 1.3[a]
Protein (45%)	4.13 ± 0.52	14.5 ± 0.5[b]	10.9 ± 1.0[b]	6.7 ± 0.4[b]
Protein (50%)	3.51 ± 0.32	15.9 ± 0.8[b]	9.6 ± 0.8[b]	8.2 ± 0.6[b]

Data are mean ± SEM, n =13.
[a-b]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table III-5 Production of CO_2 and ATP from the oxidation of nutrients by the skeletal muscle of LMB fed diets containing 40%, 45% or 50% protein

Diet	Palmitate	Glutamate	Glutamine	Glucose
CO_2 Production (nmol CO_2/mg tissue per 2 h)				
Protein (40%)	0.12 ± 0.01	0.21 ± 0.02	0.20 ± 0.01	0.16 ± 0.02
Protein (45%)	0.10 ± 0.01	0.21 ± 0.02	0.26 ± 0.03	0.18 ± 0.02
Protein (50%)	0.11 ± 0.01	0.25 ± 0.02	0.26 ± 0.03	0.17 ± 0.01
ATP Production (nmol ATP/mg tissue per 2 h)				
Protein (40%)	0.76 ± 0.08	0.96 ± 0.10	0.89 ± 0.06	0.80 ± 0.08
Protein (45%)	0.68 ± 0.07	0.95 ± 0.08	1.18 ± 0.11	0.91 ± 0.09
Protein (50%)	0.73 ± 0.07	1.13 ± 0.10	1.17 ± 0.13	0.84 ± 0.06

Data are mean ± SEM, n =13.
Means for each variable did not differ ($P > 0.05$) among fish fed the 40%, 45% and 50% protein diets.

Table III-6 Tissue weights and estimated ATP production in different tissues of a 50-g juvenile LMB

Diet		Liver	Kidney	Intestine	Skeletal muscle	Total
Tissue weight (mg)						
	Protein (40%)	2231	64	256	21000	
	Protein (45%)	1816	67	282	22000	
	Protein (50%)	931	60	266	23000	
ATP production (nmol/2h per whole tissue)						
Palmitate						
	Protein (40%)	3322 ± 206^{a}	941 ± 26^{a}	1071 ± 82	15999 ± 1694	21334 ± 1723
	Protein (45%)	2251 ± 129^{b}	841 ± 59^{ab}	1165 ± 147	15036 ± 1615	19292 ± 1640
	Protein (50%)	1229 ± 84^{c}	745 ± 54^{b}	932 ± 85	16762 ± 1646	19667 ± 950
Glutamate						
	Protein (40%)	5720 ± 298^{a}	1368 ± 76^{a}	5445 ± 183^{a}	20077 ± 2148	32611 ± 2140
	Protein (45%)	4335 ± 314^{b}	1328 ± 74^{a}	4087 ± 151^{b}	20901 ± 1820	30652 ±1848
	Protein (50%)	2668 ± 142^{c}	1114 ± 59^{b}	4211 ± 203^{b}	26372 ± 2020	34366 ± 2093
Glutamine						
	Protein (40%)	6688 ± 625^{a}	1624 ± 74^{a}	3412 ± 223^{a}	18733 ± 1357^{b}	30457 ± 1413
	Protein (45%)	4690 ± 385^{b}	1371 ± 48^{b}	3069 ± 284^{ab}	25996 ± 2510^{a}	34308 ± 2796
	Protein (50%)	3673 ± 150^{c}	1159 ± 47^{c}	2561 ± 222^{b}	26915 ± 2881^{a}	35127 ± 3039
Glucose						
	Protein (40%)	4731 ± 569^{a}	1055 ± 56^{a}	3251 ± 321^{a}	16716 ±1734	25753 ± 1841
	Protein (45%)	2620 ± 199^{b}	871 ± 41^{b}	1896 ± 116^{b}	19285 ±1912	25467 ± 2093
	Protein (50%)	1115 ± 97^{c}	644 ± 50^{c}	2179 ± 89^{b}	20090 ±1322	23223 ± 1312

Data are mean ± SEM, n =13.

$^{a-c}$: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table III-7 The growth performance and feed utilization of LMB fed for 56 days diets containing 40%, 45% or 50% protein

	40%	45%	50%
WG	157 ± 8^{b}	167 ± 5^{ab}	186 ± 7^{a}
DFI	2.08 ± 0.05	2.04 ± 0.02	2.05 ± 0.02
FCR	1.28 ± 0.06	1.20 ± 0.04	1.13 ± 0.04
Energy retention (%)	37.7 ± 1.4^{b}	40.0 ± 1.3^{b}	44.7 ± 1.2^{a}
Nitrogen retention (%)	35.7 ± 1.6	33.4 ± 1.2	33.5 ± 1.1
Lipid retention (%)	68.5 ± 2.1^{c}	81.1 ± 2.2^{b}	89.7 ± 1.9^{a}

Weight gain (%) = 100 × (final body weight - initial body weight) / initial body weight.
Feed conversion ratio (FCR) = feed intake / wet weight gain.
Daily feed intake (DFI, g/fish) = feed consumed / [(initial weight + final weight) × 0.5 × days].
Retention (or productive value, %) = 100× total gain / total intake.
Data are mean ± SEM (n = 4 tanks/diet).
[a-c]: Within a row, means not sharing the same superscript are different ($P < 0.05$).

Table III-8 Post-feeding concentrations of dispensable amino acids in the serum of LMB fed diets containing 40%, 45% or 50% protein[1].

CP	Time (h)	Asp	Glu	Asn	Ser	Gln	Gly	Cit	Tau	Ala	Tyr	Orn
40 %	0	16	32	73	186	190	351	71	831	471	79	125
	2	17	31	139	321	318	370	80	1002	617	98	147
	4	38	57	289	444	296	766	198	1174	1028	197	299
	8	23	60	266	506	252	989	243	1037	838	193	243
	12	24	61	168	518	246	707	155	1391	509	179	139
	24	20	34	99	233	253	417	78	889	489	91	127
45%	0	21	33	83	180	235	394	71	1023	500	80	134
	2	18	33	193	302	276	435	107	1132	534	148	185
	4	34	57	290	472	309	1011	173	1976	804	202	224
	8	26	54	247	501	368	1199	220	1486	1147	163	256
	12	14	50	167	517	302	463	158	1002	633	170	136
	24	19	33	72	167	179	307	51	1008	531	63	123
50%	0	23	40	85	189	201	414	58	954	451	77	157
	2	26	41	303	431	352	472	141	1176	769	213	249
	4	27	60	255	409	394	946	170	2188	863	215	251
	8	17	52	257	522	296	1059	209	1224	895	224	241
	12	17	49	190	505	340	412	122	1140	521	135	159
	24	24	45	86	211	238	415	61	951	469	91	139
Pooled SEM		0.9	1.5	11	17	8	34	8	51	26	6	7
Main effects												
	0	20^{B}	35^{B}	80^{D}	185^{D}	209^{B}	386	67^{E}	936^{D}	474^{C}	78^{C}	139^{C}
	2	20^{B}	35^{B}	212^{B}	351^{C}	315^{A}	426^{D}	109^{D}	1103^{B}	640^{B}	153^{B}	194^{B}
	4	33^{A}	58^{A}	278^{A}	442^{B}	333^{A}	908^{B}	180^{B}	1779^{A}	898^{A}	205^{A}	258^{A}
	8	22^{B}	55^{A}	256^{A}	510^{A}	305^{A}	1082^{A}	224^{A}	1249^{B}	960^{A}	194^{A}	247^{A}
	12	18^{B}	53^{A}	175^{C}	513^{A}	296^{A}	527^{C}	145^{C}	1178^{B}	555^{C}	161^{B}	145^{C}
	24	21^{B}	37^{B}	86^{D}	204^{D}	223^{B}	380^{D}	63^{E}	949^{D}	496^{C}	82^{C}	130^{C}
40%		23	46	172	368	259^{B}	601	137	1054^{B}	658	139	180^{B}
45%		22	43	175	356	278^{B}	635	130	1271^{A}	691	138	176^{B}
50%		24	48	196	378	303^{A}	620	127	1272^{A}	662	149	199^{A}
CP		0.75	0.11	0.20	0.26	<0.01	0.74	0.54	<0.01	0.32	0.19	<0.01
Time		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
CP * Time		<0.01	<0.01	0.03	0.01	<0.01	<0.01	0.21	0.22	<0.01	<0.01	0.03

[1] Values are means with pooled SEM for 4 fish per dietary group.

A-E: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Asp = aspartate; Glu = glutamate; Asn = Asparagine; Ser = serine; Gln = glutamine; Gly = glycine; Cit = citrulline; Tau = taurine; Ala = alanine; Tyr = tyrosine; Orn = ornithine

Table III-9 Post-feeding concentrations of indispensable amino acids in the serum of LMB fed diets containing 40%, 45% or 50% protein[1].

CP	Time (h)	His	Thr	Arg	Trp	Met	Val	Phe	Ile	Leu	Lys
40 %	0	136	123	24	29	60	228	97	121	226	149
	2	125	147	23	30	83	250	135	136	250	192
	4	188	356	29	36	123	603	250	289	569	260
	8	176	303	27	36	89	354	203	185	305	325
	12	130	251	26	29	70	260	216	175	247	187
	24	141	140	24	28	62	224	96	109	202	125
45%	0	111	127	26	27	55	239	94	126	218	162
	2	122	234	26	29	109	397	209	196	359	187
	4	200	350	27	32	134	531	254	250	497	280
	8	188	291	28	32	86	464	251	237	393	344
	12	150	247	24	30	63	275	179	156	243	194
	24	123	140	25	28	54	278	98	151	253	179
50%	0	119	161	24	30	57	246	95	123	217	186
	2	138	358	27	35	98	549	246	278	505	298
	4	204	359	26	28	142	591	262	303	546	321
	8	195	290	27	28	71	465	250	220	393	349
	12	169	255	27	30	60	340	132	193	281	237
	24	111	134	25	28	58	244	94	125	217	196
Pooled SEM		5	11	0.4	0.8	4	16	8	8	15	9
Main effect means											
	0	122^{C}	137^{D}	25	29^{BC}	57^{D}	237^{D}	95^{C}	123^{D}	221^{C}	166^{D}
	2	129^{C}	247^{C}	25	31^{AB}	97^{B}	399^{B}	196^{B}	204^{B}	371^{B}	226^{C}
	4	197^{A}	355^{A}	27	32^{A}	133^{A}	575^{A}	255^{A}	281^{A}	538^{A}	287^{B}
	8	186^{A}	295^{B}	27	32^{A}	82^{C}	428^{B}	235^{A}	214^{B}	363^{B}	339^{A}
	12	150^{B}	251^{C}	26	30^{AC}	64^{D}	292^{C}	176^{B}	175^{C}	257^{C}	206^{C}
	24	125^{C}	138^{D}	24	28^{C}	58^{D}	249^{D}	96^{C}	128^{D}	224^{C}	167^{D}
40%		149	220^{B}	25	31	81	320^{C}	166^{B}	169^{C}	300^{C}	206^{C}
45%		149	232^{B}	26	30	84	364^{B}	181^{A}	186^{B}	327^{B}	224^{B}
50%		156	260^{A}	26	30	81	406^{A}	180^{A}	207^{A}	360^{A}	265^{A}
CP		0.56	<0.01	0.923	<0.61	0.78	<0.01	0.04	<0.01	<0.01	<0.01
Time		<0.01	<0.01	0.30	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
CP * Time		0.129	<0.01	0.216	0.04	<0.01	<0.01	<0.01	<0.01	<0.01	0.02

[1] Values are means with pooled SEM 4 fish per dietary group.

A-E: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

His = histidine; Thr = threonine; Arg = arginine; Trp = tryptophan; Met = methionine; Val = valine; Phe = phenylalanine; Ile = isoleucine; Leu = leucine; Lys = lysine

Table III-10 Post-feeding concentrations of glucose, ammonia and lactate in the serum of LMB fed diets containing 40%, 45% or 50% protein[1].

CP	Time (h)	Glucose (mM)	Ammonia (μM)	Lactate (mM)
40%	0	4.69	59.3	1.09
	2	4.57	79.3	1.80
	4	7.23	101.4	1.88
	8	7.05	139.9	1.84
	12	8.16	71.6	2.11
	24	4.86	52.8	1.22
45%	0	4.58	62.6	0.96
	2	4.63	85.0	1.31
	4	7.93	85.8	1.82
	8	7.86	119.6	1.79
	12	8.08	95.7	1.73
	24	4.04	74.2	1.01
50%	0	4.38	83.0	1.12
	2	4.64	83.8	1.98
	4	5.45	91.9	1.67
	8	5.33	114.3	1.70
	12	5.60	92.5	1.77
	24	4.03	82.8	1.38
Pooled SEM		0.18	3.4	0.06
Main effect means				
	0	4.57^{B}	68.3^{C}	1.06^{B}
	2	4.61^{B}	82.7^{B}	1.70^{A}
	4	6.87^{A}	93.0^{B}	1.79^{A}
	8	6.75^{A}	124.6^{A}	1.78^{A}
	12	7.28^{A}	86.6^{B}	1.87^{A}
	24	4.31^{B}	69.9^{C}	1.20^{B}
40%		6.09^{A}	84.1	1.66
45%		6.19^{A}	87.2	1.44
50%		4.91^{B}	91.3	1.60
CP		< 0.01	0.59	0.12
Time		< 0.01	< 0.01	< 0.01
CP * Time		0.020	0.043	0.18

[1] Values are means with pooled SEM for 4 fish per dietary group.
A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

CHAPTER IV

EFFECTS OF DIETARY PROTEIN AND LIPID LEVELS ON GROWTH PERFORMANCE, FEED UTILIZATION, AND LIVER HISTOLOGY OF LMB (*MICROPTERUS SALMOIDESFIRST*)

Abstract Largemouth bass (LMB, native to North America) exhibit poor growth rate and pale liver syndrome when fed currently formulated diets. Because amino acids and lipids are known to affect hepatic metabolism and function in mammals, it is imperative to understand the impacts of these dietary macronutrients on both growth and liver of the fish. In this study, we designed six isocaloric diets to determine the effects of different dietary crude protein (CP; 40%, 45%, and 50%) and lipid levels (7.5% and 10%) on fat and glycogen deposits, as well as hepatosis in LMB. There were four tanks (12 fish per tank, an average initial weight of 18.4 g/fish) per dietary treatment group and the trial lasted for 8 weeks. Fish were fed to apparent satiation three times daily. Results indicated that LMB fed the 45% or 50% CP diet grew faster ($P < 0.05$), had less ($P < 0.05$) glycogen in the liver, and had smaller ($P < 0.05$) hepatocyte sizes than the fish fed the 40% CP diet, but there was no difference in weight gain or feed efficiency between the 45% and 50% CP diets. The hepatic lipid content did not differ between LMB fed the 40% and 45% CP diets, and the values for these two groups were 29% lower ($P < 0.05$) than those for LMB fed the 50% CP diet. Compared with the 40% CP group, LMB fed the 45% or 50% CP diet had 8-12% lower content of total minerals, phosphorus, and calcium in the body. Increasing the dietary lipid level from 7.5% to 10% enhanced the weight gain (+15%) and feed efficiency (+22%), as well as the retention of dietary CP (+18%), energy (+25%), and phosphorus (+7.6%) in the body. Enlargement

of the liver and hepatosis due to excessive glycogen accumulation occurred in LMB fed the 40% CP diet possibly because of high dietary starch levels (22-28%) and, to a lesser extent, in the fish fed the 45% or 50% CP diet. Liver weight did not differ between LMB fed the 7.5% and 10% lipid diets. No fatty liver occurred in any group of LMB (with hepatic lipid concentrations being < 2%). Based on these growth, metabolic and histologic data, we recommend dietary CP and lipid levels to be 45% and 10%, respectively, for juvenile LMB.

Introduction

The diets for LMB have included frozen raw fish and formulated compound feeds. Interestingly, LMB fed compound feeds frequently exhibit pale liver syndrome that contributes to their slow growth rates and mortality (Coyle et al. 2012). This metabolic disorder has been suggested to result from an excessive accumulation of glycogen (Goodwin et al. 2002) and lipids (Cardeilhac et al. 2009) in the liver. Thus, without an effective solution, formulated compound feeds have not been widely used for the farm production of LMB, which is the first limiting factor for its sustainable development (Yu et al. 2018). Interestingly, neither pale liver syndrome nor hepatosis (a functional disorder of liver) is present in LMB that are fed frozen raw fish (Goodwin et al. 2002), but there is a lack of knowledge about the underlying nutritional biochemistry. To date, high costs of frozen raw fish as well as their limited availability and potential role as a vector for disease transmission hinder the large-scale development of LMB aquaculture worldwide. Therefore, it is imperative to develop a low-cost, formulated diet that can prevent hepatic structural and functional abnormalities to support global, sustainable production of LMB.

There are reports that juvenile LMB required (per dry matter basis) 40-41% crude protein for 5 – 15 g of body weight (BW; Anderson et al. 1981), 43.6% CP for 14-23 g of BW, 51.6% CP for

10-36 g of BW (Cai et al. 2020), and 55-58% CP for 8.7-52 g of BW (Huang et al. 2017). Protein is the most expensive nutrient that influences feed and total production costs (Rahimnejad et al. 2015; Nates, 2015). Thus, the optimum requirements of LMB for dietary protein should be defined (Table I-2). At present, there is also inconsistent information about optimal requirements of LMB for dietary lipids (Chapter I), and some studies reported that formulated diets for LMB contain a wide range of lipid levels (e.g., 3.7 to 25%; Cardeilhac et al. 2009; Goodwin et al. 2002; Shi et al. 2019; Tidwell et al. 1996). High-levels of non-protein energy sources (lipids and starch) in diets can spare AAs in the diets of some fish (De Silva et al. 1991; Shiau and Peng 1993), as in land animals (Wu 2018). However, excess dietary starch and lipid levels could cause fat deposition, hepatopathy, and other health problems in fish (Asaoka et al. 2013; Han et al. 2014a), including LMB (Cardeilhac et al. 2009; Goodwin et al. 2002; Shi et al. 2019). Because AAs and lipids in diets play important roles in hepatic metabolism and function (Wu 2018), it is imperative to understand the impacts of these dietary macronutrients and their interactions on both the growth and the liver function of the fish.

At present, little is known about the effects of dietary protein and lipids or their interactions on hepatic glycogen in LMB (e.g., Bright et al. 2005; Cai et al. 2020; Zhou et al. 2020). In addition, there are conflicting reports [e.g., no effect (Huang et al. 2017), reductions (Chen et al. 2012), or increases (Portz et al. 2001)] regarding the effects of dietary protein levels on nitrogen and lipid retention in LMB. Thus, the current study was conducted with six isocaloric diets to determine the effects of different dietary CP (40%, 45%, and 50%) and lipid levels (7.5% and 10%) and their interactions on fat and glycogen deposits, as well as hepatosis in LMB.

Materials and Methods

Experimental Diets

Six isocaloric experimental diets were formulated to contain three CP levels (40%, 45%, and 50%) and two lipid levels (7.5% and 10%) with starch ranging from 9.20% to 28.1% to obtain isocaloric diets. The ingredients and proximate composition of diets are shown in Table IV-1. The gross energy content of the diets was calculated based on 22.6 kJ/g, 39.3 kJ/g and 17.2 kJ/g for protein, lipid and glycogen/starch, respectively (Wu 2018). The main protein sources used in this study were fish meal and soybean protein concentrate with the defined composition of AAs, carbohydrate and lipids (Li and Wu 2020). The crude lipid levels were adjusted by using fish oil, poultry fat, and soybean oil. All ingredients were thoroughly mixed using a mixer, and thereafter, fish oil, soybean oil, and water were added to the mixture to form a moist dough. The experimental diets were produced by a screw extruder (Big Bite Meat Grinder, West Chester, OH) and oven-dried at 50 ℃ until dry matter content was 97.0%. All feeds were kept at – 20 ℃.

Experimental Animals

Juvenile LMB were obtained from a commercial fish farm (Larry's Fish Farm, Giddings, TX, USA) and housed in the Kleberg Center of Texas A&M University, as previously described (Jia et al. 2017). The photoperiod of the housing facility was maintained for 14 h per day, with lights being turned off between 10:00 PM and 8:00 AM. Prior to starting the experiments, LMB were acclimated to the experimental condition and maintained on a commercial diet (AquaMax® Grower 400, Purina, MO) for 2 weeks. The experimental system consisted of 18 tanks, three tanks for each treatment. Each tank contained 55 L water (26 ^{o}C). At the beginning of this trial, 18 fish with a uniform body size (initial body weight, ~18.3 g per fish) were randomly distributed into

each tank. The mean BW of the fish did not differ ($P > 0.05$) among the different treatment groups. Air was supplied to the water through air stones connected to air pumps, with its salinity being maintained at 2-5 ppt. The quality parameters of water [pH 6.5-7.5, NH_4^+ (< 1 mg/L), nitrite (< 1 mg/L), nitrate (< 20 ppm), and dissolved O_2 (8 ppm)] were monitored daily and remained within acceptable limits. Fish were hand-fed with experimental diets to apparent satiation three times daily at 09:00, 15:00 and 20:00. Total feed consumption was recorded every day. We determined that the loss of provided pellet feed into the water was 10%, and therefore, the true feed consumption by fish was calculated as the amount of provided feed x 0.9. Total fish weight in each tank was recorded every two weeks to minimize handling and stress. Fish were weighed after a 24-h period of food deprivation. Almost 100% water in tank was replaced daily. The feeding trial lasted for 56 days.

Sample collection

At the beginning of the trial, 30 fish were euthanized with 140 ppm MS-222 (neutralized by an appropriate amount of $NaHCO_3$) for the analysis of whole-body composition. At the end of the experiment, blood (0.5 ml) was collected from the caudal vein of conscious fish (6 fish/tank) with the use of a hypodermic syringe at various time points after feeding. After blood collection, all fish were euthanized as described previously, with four fish being randomly selected from each tank for the analyses of whole-body composition and with five fish being randomly selected from each tank to obtain viscera organs [including the stomach and intestine (without luminal contents), as well as liver, pancreas, and spleen)], peritoneal adipose tissue, and skeletal muscle. Blood samples were immediately centrifuged (2 min at 8000 g) at 4 ℃. The supernatant fluid (serum) was obtained and stored at -80 ℃ until analyzed.

Biochemical analyses

CP (N × 6.25) was determined by the combustion method and calcium and phosphorus were determined by optical spectrometry in Servi-Tech laboratories (Amarillo, TX, USA). Moisture was determined by drying at 105 ℃ in an oven to a constant weight. Lipids were extracted from the samples with chloroform/methanol (2:1 v/v) according to the method of Folch et al. (1957). Glucose in neutralized samples was determined enzymatically using a fluorometric method involving hexokinase and glucose-6-phosphate dehydrogenase (Fu et al. 2005), and lactate was analyzed by using lactate dehydrogenase (Wu et al 1995). Gross energy contents were calculated based on 22.6 kJ/g, 39.3 kJ/g and 17.2 kJ/g for protein, crude lipid, and glycogen/starch, respectively.

Serum metabolites and enzyme assays

Glucose in deproteinated serum was analyzed by using a fluorometric method involving hexokinase and glucose-6-phosphate dehydrogenase (Fu et al, 2005). Aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured enzymatically by using fluorometric methods at 340 nm at 25 ℃ (Wu et al. 2000). One unit of AST or ALT activity is defined as the transamination of one micromole of L-aspartate or L-alanine per min at 25℃ and pH 7.5. Lactate dehydrogenase (LDH) was measured by using the fluorometric method as described by Wu et al. (1995). One unit of LDH activity is defined as one micromole of pyruvate converted into L-lactate per min at 25℃ and pH 7.0. Alkaline phosphatase (ALP) activity was measured by a fluorometric method as described by Sabokbar et al. (1994) with some modifications. One unit of ALP is defined as one micromole of p-nitrophenol produced from p-

nitrophenyl-phosphate per min at 25 °C and pH 9.8. The assays of all the enzymes were linear with time (0 to 20 min) and the amount of tissue protein (0.1 to 0.5 mg/ml) used in the solutions.

Histological analysis of the liver

A portion (500 mg) of the liver was fixed with 4% paraformaldehyde (buffered to pH 7.2) for 24 h. The samples were rinsed, dehydrated, and embedded in paraffin for histological analysis at the Veterinary Medicine & Biomedical Sciences Histology Laboratory of Texas A&M University. Each sample was cut into two of 6 μm transverse sections with a rotary microtome. The sections were stained with the periodic acid-Schiff (PAS) solution as described by Fu and Campbell-Thompson (2017). One section was digested by α-amylase, and the other was treated with the vehicle buffer, and both were stained with the PAS solution. The PAS solution containing diastase (an enzyme that digests glycogen) was also used to stain the tissue for differentiating glycogen from other PAS-positive elements in tissue. The sizes of hepatocytes were measured as their areas in the images of livers from five fish/tank (10 cells per liver image). The liver images were evaluated by using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axiocam HR digital camera. The areas of hepatocytes were calculated by using the Axiovision 4.3 software of the instrument.

Calculation and statistical analysis

Growth performance and feed utilization were calculated as follows:

Weight gain (WG, %) =100 × (final body weight - initial body weight)/initial body weight;

Feed intake (FI, g/fish) = total feed intake by all fish in a tank/number of fish in the tank

Feed conversion ratio (FCR) = feed intake/body weight gain;

Protein efficiency ratio (PER) = body weight gain/dietary protein intake;

Viscerosomatic index (VSI, %) = 100 × (viscera weight/whole body weight);

Hepatosomatic index (HSI, %) = 100 × (liver weight/whole body weight);

Intraperitoneal fat ratio (IPFR, %) = 100 × (intraperitoneal fat weight/whole body weight);

Retention or productive value of dietary nutrient and energy (%) = 100 × nutrient or energy gain/dietary nutrient or energy intake. The term "productive value" is used because some of the lipids gained in animals (including fish) are derived from de novo synthesis.

All data were tested for homogeneity (the Levene's test) and normal distribution (the Kolmogorov–Smirnov test). When the tests were statistically significant, the data were log transformed before analysis. The log transformed data were normally-distributed. A 2×3 factorial analysis of variance (ANOVA) was used to analyze the main effects (lipids and protein) and their interactions. The tank for fish rearing was the experimental unit for data analyses. Differences among treatment groups were analyzed by the Student-Newman-Keuls multiple comparison test. *P*-values＜0.05 were taken to indicate statistical significance. Survival data were analyzed by the Chi square analysis**.** All statistical analyses were performed using the SPSS package (version 18.0, Chicago, IL).

Results

Growth and survival rates of LMB

The body weights and the percentage of weight gains of LMB fed different diets are presented in Table IV-2. Except for day 14, on each day of measurement during the 8-week experimental period, the body weights and the percentage of weight gains (WG) did not differ ($P > 0.05$) between LMB fed the 45% and 50% CP diets, and the values for these two groups of fish were greater ($P < 0.05$) than those for LMB fed the 40% CP diet. On day 14 of the trial, the body weight

of LMB fed the 40% CP diet was lower ($P < 0.05$) than that of LMB fed the 45% CP diet and was not different ($P > 0.05$) from that in fish fed the 50% CP diet. On each day of measurement during the 8-week experimental period, increasing the dietary lipid content from 7.5% to 10% enhanced ($P < 0.05$) body weights and the percentage of weight gains of LMB. On day 42, there were interactions ($P < 0.05$) between dietary CP and lipid levels in affecting the body weight of LMB, in that the fish fed the 50% CP and 10% lipid diet had the highest ($P < 0.05$) body weight among all the treatment groups. On days 42 and 56, there were interactions ($P < 0.05$) between dietary protein and lipid levels in affecting the percentage of weight gains in LMB. Specifically, the percentage of weight gains in LMB fed the 50% CP and 10% lipid diet was the highest ($P < 0.05$) among all the treatment groups.

The rates of survival of LMB fed the 40%, 45% and 50% CP diets were 93.6%, 96.9%, and 93.6%, respectively, whereas the rates of survival of LMB fed the 7.5% and 10% lipid diets were 95.1% and 94.4, respectively (Table IV-4). Increasing the dietary CP level from 40% to 50% or the dietary lipid level from 7.5% to 10% did not affect ($P > 0.05$) the survival of LMB. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the survival of the fish.

Feed intake, feed conversion ratio (FCR) and protein efficiency ratio (PER) of LMB

Data on the feed intake (g/fish), FCR, and protein efficiency ratio (PER) are summarized in Table IV-3. During day 0 to day 42, there was no difference ($P > 0.05$) in feed intake among LMB fed the 40%, 45% and 50% CP diets. Between days 42 and 56 of the trial, feed intake did not differ ($P > 0.05$) between LMB fed the 45% and 50% CP diets, and the values for these two groups of fish were greater ($P < 0.05$) than those for LMB fed the 40% CP diet. During the entire 56-day

experiment, feed intake (g/fish) did not differ ($P > 0.05$) among the fish fed the 40%, 45% and 50% CP diets. Increasing the dietary lipid level from 7.5% to 10% did not affect the feed intake of LMB between days 0 and 28 of the trial, but increased ($P < 0.05$) their feed intake during days 28-42 and 42-56 by 7.3%% and 7.8%, respectively. During the entire 56-day experiment, feed intake (g/fish) was 4.1% higher ($P < 0.05$) in fish fed the 10% lipid diet, compared with fish fed the 7.5% lipid diet. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the feed intake of LMB during the entire 56-day experimental period.

The effects of dietary protein and lipid levels on the FCR varied with days (Table IV-3). Specifically, during the first 2 weeks of the trial, the FCR did not differ ($P > 0.05$) between LMB fed the 45% and 50% CP diets, and the values for these two groups of fish were 17.8% and 11.1% lower ($P < 0.05$) than those for LMB fed the 40% CP diet, respectively. However, during the second 2 weeks of the trial, the FCR did not differ ($P > 0.05$) between LMB fed the 40% and 45% CP diets, and the values for these two groups of fish were higher ($P < 0.05$) than those for LMB fed the 40% CP diet. Between days 28 and 56, the FCR did not differ ($P > 0.05$) among the three groups of fish. In contrast, increasing the dietary lipid level from 7.5% to 10% decreased ($P < 0.05$) the FCR of LMB during days 0-14 and 14-28 by 11%% and 10%, respectively, had no effect ($P > 0.05$) on the FCR during days 28-42, and reduced decreased ($P < 0.05$) the FCR by 21.5%. On days 42-56, there were interactions ($P < 0.05$) between dietary CP and lipid levels in affecting the FCR of LMB. Specifically, LMB fed the 40% CP and 7.5% diet and the 45% CP and 7.5% lipid diet had the highest ($P < 0.05$) FCR among all the treatment groups. However, between days 0 and 42 of the trial, there were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the FCR of LMB.

The effects of dietary protein and lipid levels on the PER of LMB varied with days (Table IV-3). Specifically, during the first 2 weeks of the trial, the PER did not differ ($P > 0.05$) between LMB fed the 40% and 45% CP diets, and the values for the 45% CP group were 20% higher ($P < 0.05$) than those for LMB fed the 50% CP diet. On days 28-42, the PER did not differ ($P > 0.05$) between LMB fed the 40% and 45% CP diets, and the values for the 40% CP group were 25% higher ($P < 0.05$) than those for LMB fed the 50% CP diet. During days 14-28 and 42-56 of the trial, the PER of LMB was not affected ($P > 0.05$) by dietary protein levels. In contrast, increasing the dietary lipid level from 7.5% to 10% increased ($P < 0.05$) the PER during days 0-14, 14-28, 28-42, and 42-56 by 17.3%, 11.3%, 8.4%, and 27.7%, respectively. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the PER of LMB during the entire experimental period.

Relative weights of all viscera organs, liver, and intraperitoneal adipose tissue of LMB

Data on the VSI, HSI, and IPFR of LMB fed different diets are shown in Table IV-4. Viscera organs, liver, and IPFR represented 7.0-9.2%, 2.1-4.3%, and 1.6-1.9% of the whole-body weight, respectively. The hepatocyte size ranged from 115 to 339 μm^2. Increasing the dietary protein level from 40% to 50% progressively decreased ($P < 0.05$) the VSI, HSI, hepatocyte size in LMB. The IPFR did not differ ($P > 0.05$) between LMB fed the 45% and 50% CP diets, and the values for these two groups of fish were greater ($P < 0.05$) than those for LMB fed the 40% CP diet. In contrast, increasing the dietary lipid level from 7.5% to 10% did not affect ($P > 0.05$) the VSI or HSI, reduced ($P < 0.05$) the hepatocyte size by 7.6%, and enhanced ($P < 0.05$) the IPFR by 22.8%. There were interactions ($P < 0.05$) between dietary protein and lipid levels in affecting the VSI, HSI, and hepatocyte size. Specifically, LMB fed the 40% CP and 7.5% diet, the 40% CP and 10%

lipid diet, and the 45% CP and 10% lipid diet had the highest ($P < 0.05$) VSI among all the treatment groups; LMB fed the 40% CP and 7.5% lipid diet, the 40% CP and 10% lipid diet had the highest ($P < 0.05$) HSI among all the treatment groups; and LMB fed the 40% CP and 7.5% lipid diet had the highest ($P < 0.05$) hepatocyte size among all the treatment groups. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the IPFR of LMB.

Composition of macronutrients in the liver, skeletal muscle, and the whole body of LMB

At the end of the 56-day trial, the absolute amounts of water, protein, lipids and glycogen were lower ($P < 0.05$) in LMB fed the 50% CP diet than those in LMB fed the 40% CP diet (Table IV-5), which was consistent with the reduced mass of the liver or the HSI (Table IV-4). Increasing the dietary lipid level from 7.5% to 10% did not affect ($P > 0.05$) the absolute amounts of water, protein, or glycogen in the liver of LMB, but increased ($P < 0.05$) the absolute amounts of lipid in the liver by 15.4%. There were interactions ($P < 0.05$) between dietary starch and lipid levels in affecting the absolute amounts of water, lipid, and glycogen in the liver. Specifically, the amount of water, lipids or glycogen in the liver of LMB fed the 40% CP and 10% lipid diet was the highest ($P < 0.05$) among all the dietary groups. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the amount of protein in the liver.

The content of water, protein, lipids, and glycogen in the liver of LMB was about 68-69%, 7.1-10.7%, 1.3-1.8%, and 12.7-14.9%, respectively (Table IV-5). Increasing the dietary CP from 40% to 50% did not affect ($P > 0.05$) the hepatic content of water, increased ($P < 0.001$) the hepatic concentrations of protein (+50.5%) and lipids (+44.9%), and decreased the hepatic concentration of glycogen (-14.8%). Intrahepatic concentrations of protein and glycogen did not differ ($P > 0.05$) between LMB fed the 45% and 50% CP diets. Increasing the dietary lipid level from 10% to 12.5%

did not affect ($P > 0.05$) the content of water, protein, lipids or glycogen in the liver of LMB. There were interactions ($P < 0.05$) between dietary starch and lipid levels in affecting the intrahepatic concentrations of lipids. Specifically, the content of lipids in the liver of LMB fed the 50% CP and 7.5-10% lipid diets was the highest ($P < 0.05$) among all the dietary groups. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the intrahepatic content of water, protein and glycogen in LMB.

The content of water, protein, lipids, and glycogen in the skeletal muscle of LMB was about 77%, 20%, 1.0-1.1%, and 12.7-14.9%, respectively (Table IV-6). Increasing the dietary CP level from 40% to 50% or the dietary lipid level from 7.5% to 10% did not affect ($P > 0.05$) intramuscular content of water or protein in LMB. In contrast, increasing the dietary CP level from 40% to 50% decreased ($P < 0.05$) the intramuscular content of lipids and glycogen by 8% and 21.5%, respectively, whereas increasing the dietary lipid level from 7.5% to 10% increased ($P < 0.05$) intramuscular content of lipids by 8%, but did not affect ($P > 0.05$) the intramuscular content of glycogen in LMB. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the intramuscular content of water, protein, lipids and glycogen in LMB.

The whole body of LMB contained 69-70% water, about 18% protein, 5.2-6.5% lipids, and 4.9-5.5% total minerals (including 0.86-0.96% phosphorus and 1.4-1.6% calcium) (Table IV-6). Increasing the dietary CP level from 40% to 50% did not affect ($P > 0.05$) the content of water, protein, or lipids in the whole body. Compared with the 40% CP group, LMB fed the 45% or 50% CP diet had a lower ($P < 0.05$) content of total minerals, phosphorus, and calcium in the body. Increasing the dietary lipid level from 7.5% to 10% did not affect ($P > 0.05$) the content of water, protein, or total minerals in the whole body, increased ($P < 0.05$) the content of lipids in the whole

body by 24.7%, and decreased ($P < 0.05$) the content of phosphorus and calcium in the whole body by 6.5% and 12.2%, respectively. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the content of water, protein, lipids, and total minerals (including phosphorus and calcium) in the whole body of LMB.

Retention (or productive value, %) of dietary energy, CP, lipids, phosphorus, calcium and amino acids in the body of LMB

During the entire 56-day trial, 29% to 37% of dietary energy, 29% to 33% of dietary CP, 66% to 73% of dietary lipids, 36% to 49% of dietary phosphorus, and 43% to 59% of dietary calcium were retained in the body of LMB (Table IV-7). Increasing the dietary CP level from 40% to 50% decreased ($P < 0.05$) the retention of dietary CP, phosphorus and calcium in the body of LMB, but increased the retention of dietary energy and lipids in the body. Increasing the dietary lipid level from 7.5% to 10% did not affect ($P > 0.05$) the retention of lipids and calcium in the body of LMB but increased ($P < 0.05$) the retention of dietary CP, energy, and phosphorus in the body by 17.9%, 25.2%, and 7.6%, respectively. There were interactions ($P < 0.05$) between dietary CP and lipid levels in affecting the retention of dietary lipids and energy in the whole body of LMB. Specifically, LMB fed the 50% CP and 10% lipids had the highest ($P < 0.05$) retention of dietary lipids and energy in the body among all the dietary groups. However, there were no interactions ($P > 0.05$) between dietary CP and lipid levels in affecting the retention of dietary CP, phosphorus and calcium in the whole body of LMB.

Data on the percentages of the gains of nutritionally dispensable and indispensable amino acids in the body of LMB to their dietary intakes are summarized in Tables IV-8 and 9, respectively. Increasing the dietary CP level from 40% to 50% decreased ($P < 0.05$) the retention of most amino

acids in the body of LMB, except for asparagine, aspartate, glutamate, glutamine, serine, and valine. In contrast, increasing the dietary lipid level from 7.5% to 10% increased ($P < 0.05$) the retention of most amino acids, except for alanine, serine, tyrosine, arginine, isoleucine, and valine. There were interactions ($P < 0.05$) between dietary protein and lipid levels in affecting the percentages of the gains of all dietary dispensable amino acids in the body of LMB, except for proline and 4-hydroxyproline. Interestingly, there were no interactions between dietary protein and lipid levels in influencing the retention of all dietary dispensable amino acids in the body of LMB.

Concentrations of glucose in the serum of LMB

Concentrations of glucose in the serum of LMB were 4.53, 6.68, 6.82, 6.56, and 4.10 mM (pooled SEM = 0.14; n = 24 tanks per time point) at 2, 4, 8, 12, and 24 h after feeding. The concentrations of glucose in serum increased ($P < 0.001$) progressively between 2 and 4 h after feeding, remained at elevated levels between 4 and 12 h after feeding, and thereafter decreased ($P < 0.001$) progressively between 12 and 24 h after feeding. Increasing the dietary CP level from 40% to 50% decreased ($P < 0.001$) the concentrations of glucose in serum at 4 to 12 h after feeding. In contrast, the effect of dietary lipids on the concentrations of glucose in serum was time-dependent. Specifically, increasing the dietary lipid level from 7.5% to 10% decreased ($P < 0.05$) the concentrations of glucose in serum by 20% at 8 h after feeding, and increased ($P < 0.05$) by 23% and 17% at 12 and 24 h, respectively. At 12 and h after feeding, there were interactions ($P < 0.05$) between dietary starch and lipid levels in affecting the concentrations of glucose in the serum of LMB. Specifically, at 12 h after feeding, the concentrations of glucose in serum was highest in LMB fed the 40% CP and 10% lipid diet and the 45% CP and 10% lipid diet among all the dietary

groups; at 24 h after feeding, the concentrations of glucose in serum was highest in LMB fed the 40% CP and 10% lipid diet, the 45% CP and 10% lipid diet, and the 50% CP and 7.5% lipid diet among all the dietary groups.

Liver histology

Histological analyses revealed that, compared with the 50% CP diet, LMB fed the 40% and 45% CP diets exhibited enlarged and pale hepatocytes with more glycogen in the cytosol, as indicated by the PAS stain (Fig. IV-2). These histologic findings are characteristics of glycogenic hepatopathy (hepatic glycogenosis). Increasing the dietary CP level from 40% to 50% progressively decreased ($P < 0.001$) the hepatocyte size by 66.1%, and increasing the dietary lipid level from 10% to 12.5% decreased ($P < 0.001$) the hepatocyte size by 7.6%. There were interactions ($P < 0.05$) between dietary starch and lipid levels in affecting the hepatocyte size of LMB. Specifically, the hepatocyte size in the liver of LMB fed the 50% CP and 10% lipid diet was the smallest ($P < 0.05$) among all the dietary groups,

Activities of enzymes in the serum and liver of LMB

The activities of aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) activities in the serum and liver of LMB fed different diets are presented in Table IV-9. Increasing the dietary CP level from 40% to 50% increased ($P < 0.05$) the activities of AST in the serum and liver of LMB as well as hepatic ALT activity, had no effect ($P > 0.05$) on serum ALT activity, and decreased ($P < 0.05$) the activities of LDH and ALP in the serum and liver. In contrast, increasing the dietary lipid level from 7.5% to 10% did not affect ($P > 0.05$) the activity of AST, LDH and ALP in serum but increased ($P < 0.05$) serum ALT activity by 13.7%. The hepatic activity of AST was 19.3% higher ($P < 0.05$) but that

of ALP was 15.7% lower ($P < 0.05$) in LMB fed the 10% lipid diet than those in LMB fed the 7.5% lipid diet. The hepatic activity of ALT or LDH did not differ ($P > 0.05$) between LMB fed the 7.5% and 10% lipid diets. There were no interactions ($P > 0.05$) between dietary protein and lipid levels in affecting the activities of all the measured enzymes in the serum and liver of LMB.

Discussion

As a carnivore, LMB have different requirements for dietary protein, carbohydrate and lipids than omnivores and herbivores (Coyle et al. 2012; Zhong et al. 2020; Zhou et al. 2020). Results of this dissertation research indicated that glutamine, glutamate and aspartate were the major metabolic fuels for the proximal intestine, liver, kidney, and skeletal muscle of LMB (Chapter II). Based on the patterns of oxidation of nutrients between LMB (Chapter II) and hybrid striped bass (another carnivore; Jia et al. 2017), it is possible that the expression of enzymes related to the metabolism of AAs, glucose and fatty acids may differ between LMB and other carnivores. At present, little is known about the optimal requirements of LMB for dietary protein and lipids, because the published data from different studies varied greatly (Table I-2). The current study was conducted to fill this critical gap of knowledge in our understanding of nutrition and metabolism in LMB.

The major findings of this work are that: (1) LMB fed the 45% or 50% CP diet grew faster, deposited less glycogen in the liver, and had smaller hepatocyte sizes than the fish fed the 40% CP diet without affecting protein composition or feed efficiency. (2) Compared with the 40% CP group, LMB fed the 45% or 50% CP diet had 8-12% lower content of total minerals (including phosphorus and calcium) in the body. (3) Increasing the dietary lipid level from 7.5% to 10% enhanced the weight gains and feed efficiency of LMB, as well as the retention of dietary protein,

energy, amino acids, and phosphorus in the body, but did not affect hepatic lipid concentrations. (4) Enlargement of the liver and hepatosis due to excessive glycogen accumulation occurred in LMB fed the 40% CP diet but were attenuated in the fish fed 45% or 50% CP diet. Thus, dietary protein and lipid levels can profoundly influence the growth, metabolism and hepatic histology of juvenile LMB, as well as the retention of most amino acids in the body of LMB. Based on the growth performance and feed efficiency of LMB reared under our experimental conditions, an optimal its requirement for dietary CP is 45% of dry matter in diet. This is similar to a previous report that minimum CP requirement of 14-23 g LMB was 44% of the dry diet (Portz et al. 2001). However, Anderson et al. (1981) found that 40-41% CP in diets (containing 10% lipids) promoted maximal growth of LMB (1.8 to 6.7 g or 5 to 15 g). This CP level may be inadequate for modern breeds of LMB. In contrast, recent results indicated that optimal CP levels for the diets of juvenile LMB (8.7 to 52 g) were either 51.6% of the dry diet containing 12.2% lipids (Cai et al. 2020) or 55-58% of the dry diet containing 13.6% lipids (Huang et al. 2017). These different recommended levels could be mainly attributed to differences in the body weights of LMB, protein sources used in compound feed formulation, and dietary levels of lipids and carbohydrate.

Nutritional strategies should help to maximize the commercial value of aquaculture production when reserchers develop compound feeds for fish species, while reducing the excretion of nitrogenous wastes from the fish into the surrounding environment (Wang et al. 2005). Liver size is highly sensitive to nutritional status in many fishes, which can be directly affected by the quantity and quality of feed and its nutrient composition (Hou et al. 2020). In this study, we found that LMB fed 40% CP diets had a higher HSI and, thus, a higher VSI, in comparison with LMB fed 45% or 50% CP diets (Table IV-4). This result suggests that diets containing ≤ 40% CP (dry

matter basis) are suboptimal for the growth and hepatic health of LMB. Because AAs act in concert with lipids to fulfil their nutritional and physiological functions, dietary lipid levels influence the dietary protein requirements of any species, including fish (Wu 2018). This view is further supported by our finding that dietary protein and lipids interacted to affect the weight gains (Table IV-2), feed efficiency (Table IV-3), VSI and HSI (Table IV-4), hepatocyte size (Table IV-4), liver (Table IV-5) and body (Table IV-6) composition, energy and nutrient retention (Table IV-7), and serum glucose concentration (Table IV-10) in LMB. Thus, because traditional compound feeds for LMB contained a wide range of lipid levels (e.g., 3.7 to 25%; Cardeilhac et al. 2009; Goodwin et al. 2002; Shi et al. 2019; Tidwell et al. 1996), it is imperative to identify optimal requirements of this fish for dietary lipids. In the current work, we found LMB fed 10% lipid diets exhibited better growth performance and feed utilization efficiency than those fed 7.5% lipid diets (Table IV-3). Results of our another study indicated that LMB fed 10% lipid diets exhibited higher growth and survival rates than the fish fed 12.5% lipid diets (Chapter IV). Thus, we recommend a dietary lipid level of 10% (dry matter basis) for juvenile LMB, as suggested by other researchers (Anderson et al. 1981; Portz et al. 2001; Zhou et al. 2020).

The pale liver syndrome (including liver enlargement and dysfunction) in LMB fed compound feeds was recognized in the 2000s as a major factor causing reduced feed intake and slow growth and as well as high morbidity and mortality in this fish (Goodwin et al. 2002). High HSI values are often related to the fish's poor growth and health (Li et al. 2014). Results of this study revealed that this metabolic disorder is not caused by excessive lipids in hepatocytes but rather results from excessive glycogen accumulation in the liver of LMB. Low protein levels could induce the enlargement of hepatocytes filled with excessive glycogen and water (3 g water/g glycogen;

Brooks and Fahey 1984), which leads to hepatosis. In LMB fed 40% CP diets, the concentrations of glucose in serum and the concentrations of glycogen in the liver were much higher than those in the fish fed 45% and 50% CP diets (Tale IV-10). This may be explained by the following reasons. First, the 40% CP diets contained more starch content than the 45% and 50% CP diets (Table IV-1). Second, LMB have a limited ability to oxidize glucose in its tissues (including the liver, skeletal muscle, and kidneys) (Chapter II and III); therefore, the higher circulating levels of glucose for a prolonged period (within 12 h after feeding; Table IV-11) promote hepatic glycogen synthesis. Third, the synthesis and storage of glycogen in the skeletal muscle of LMB are limited due to the low uptake of glucose (Li and Wu 2019). Thus, this organ cannot compensate for a low rate of glucose utilization in the fish. Fourth, reduced intake of dietary protein is expected to impair the hepatic production of nitric oxide (NO) from L-arginine (Wu and Meininger 2002), which is an abundant AA in fishmeal (Li and Wu 2020). NO is a signaling molecule to regulate glycogen metabolism in hepatocytes (Jobgen et al. 2006). Specifically, NO inhibits glycogen synthesis in hepatocytes by suppressing the conversion of glycogen synthase b (inactive form) into glycogen synthase a (active form) (Sprangers et al. 1998), while promoting glycogenolysis in hepatocytes through the activation of glycogen phosphorylase (Borgs et al. 1996). Such biochemical mechanisms may explain, in part, why LMB fed raw fish do not develop glycogenosis or hepatosis (Coyle et al. 2012).

All fish, including the LMB, have physiological requirements for glucose (Tidwell et al. 2019). However, a high level of digestible carbohydrate (e.g., starch) in diet negatively affects the morphology and health of the LMB's liver and, therefore, is expected to influence the requirement of the fish for dietary protein. In the study of Huang et al. (2017), the growth rate of the juvenile

LMB was higher when fed diets with 48-51% CP and < 14% starch (as-fed basis), in comparison with the fish fed diets with 42-45% CP and > 18% starch (as-fed basis). We cannot exclude a possibility that the lower starch level in the 48% and 50% CP diets may contribute to the better growth of the fish, but Huang et al. (2017) made no discussion on this issue in their work. In our current study, we found that: (1) compared with the LMB fed the 50% CP (DM basis) diet containing 12-15% fiber [cellulose plus sodium carboxy methyl cellulose (the binder)], the LMB fed diets with 45% CP (DM basis) had similar growth performance (Table IV-2), FCR (Table IV-3), and PER (Table IV-3), as well as higher rates of the utilization of dietary AAs for protein accretion in the body (Tables IV-8 and 9). Note that our FCR values (feed intake/weight gain; 1.17 to 1.40) for the juvenile LMB during the entire 8-week experimental period are very similar to those reported by other researchers (e.g., Guo et al. 2019; Song et al. 2018; Zhou et al. 2020; Zhong et al. 2020) for juvenile LMB with similar BW. Thus, the dietary content of 45% CP (DM basis) is sufficient for juvenile LMB (18 to 50 g).

Lipids are major components of animals and also serve as key molecules for the regulation of nutrient metabolism (Wu 2018). Adequate intake of dietary lipids is crucial for the growth, metabolism, protective function, and survival of animals, including fish (Smith 2013; Smith and Prior 1986). A salient observation from the current work is that increasing the dietary lipid content from 7.5% to 10% increased the content of intraperitoneal adipose tissue by 23%, did not affect the concentrations of lipids in the liver of LMB (Table IV-5), and only slightly enhanced intramuscular lipids by 8% (Table IV-6). These patterns of metabolic responses in LMB differ very much from those in rats (Jobgen et al. 2009), where augmenting dietary intake of lipids promoted a marked increase in not only intraperitoneal adipose tissue but also intrahepatic and

intramuscular lipids. The rate of oxidation of fatty acids in the liver and skeletal muscle of LMB was very low (Chapter II). Thus, the nutritional and physiological regulation of depot-specific metabolism of lipids likely differs between LMB and mammals. Hepatic steatosis hepatopathy is a common metabolic disorder in swine and poultry fed a large amount of dietary fats (Wu 2020). In humans, nonalcohol fatty liver disease (NAFLD), which is defined by the presence of macrovesicular fat accumulation in more than 5% of hepatocytes, also occurs in response to high fat intake (Yuan and Bambha, 2015). As for glucose, the tissues of LMB, such as the liver and skeletal muscle, have a limited ability to oxidize fatty acids (Chapter II). Thus, LMB fed compound feeds containing very high lipid content (e.g., ≥ 25% fats), hepatic steatosis occurs because of the direct deposition of dietary lipids in the liver (Goodwin et al. 2002). At present, there is no criterion for the diagnosis of fatty liver in fish, because of species differences in normal lipid concentrations under physiological conditions. For example, lipid content can be up to 70% in the liver of Atlantic cod (*Gadus morhua*) without causing growth restriction or hepatic dysfunction, while Atlantic salmon (*Salmo salar*) only has about 10% of hepatic lipid (NRC, 2011). Interestingly, the intrahepatic concentration of lipid in all groups of LMB was less than 2%, further supporting the notion that within certain dietary levels (e.g., 10-12.5%), a fatty liver does not develop in this fish. This is consistent with the liver histology of LMB fed different diets (Fig IV-1).

There are recommendations that lipids or carbohydrates be used in the diets of fish as energy sources to minimize the use of high-priced protein as an energy source (Amoah et al. 2008; Garling and Wilson 1976; Wang et al., 2017). However, this study indicated that low dietary protein to energy ratio with a high dietary starch level could induce hepatosis, which further restricts the fish growth performance. For LMB, the dietary protein, lipid, and starch levels were designed as 45-

50%, 10-13%, and 20%, respectively (Chen et al. 2012 a, b; Chen et al. 2015; Yu et al. 2018). In these studies, the ratio of dietary protein to energy is only about 20 mg kJ^{-1}, which is much lower than the optimal value from some carnivorous fish. For grouper, fish fed a diet containing about 30 mg protein per kJ energy exhibited the best growth performance (Wang et al. 2017; Le and Williams 2007). Similarly, this study indicated that LMB fed diet P50/L10 containing 29.6 mg protein per kJ energy exhibited the best growth performance. However, fish fed the diet with the same ratio of protein to energy that contained 50% protein and 7.5% lipids exhibited lower growth performance than fish fed the P50/L10 diet. This result indicated that the concept of dietary protein:energy ratio (Garling and Wilson 1976) should be used with caution when diets are formulated for the LMB.

Dietary energy is converted into biological energy when nutrients are metabolized in animals (Tidwell et al. 2019; Wu 2018). Thus, researchers and producers must consider the dietary content of all macronutrients that serve as energy substrates in animals (including fish), i.e., protein, lipids, and starch. All fish have physiological requirements for glucose. Although the animals can synthesize glucose from AAs and other glucogenic precursors (e.g., glycerol, pyruvate and lactate), the inclusion of starch can spare dietary protein in some fish (Amoah et al. 2008). To minimize the use of high-priced protein aquafeeds, it has been recommended that they provide adequate carbohydrates (Wang et al. 2017). There are suggestions that the diets for juvenile LMB contain 20% starch (dry matter basis) in addition to 45-50% CP and 10-13% lipids (Chen et al. 2012 a, b; Chen et al.2015; Yu et al. 2018). In these studies, the ratio of dietary CP to protein was only about 20 mg kJ^{-1}, which is much lower than the optimal value from some carnivorous fish, such as 30 mg kJ^{-1} in the diets of grouper (Wang et al. 2017, 2017; Le and Williams 2007). Similarly, results

of our present study indicated that LMB fed the diet containing 50% CP, 10% lipids and 9.2% starch (providing the value of 30.1 mg kJ^{-1} for the ratio of CP to energy in diet) exhibited better growth performance than other groups of the fish (Table IV-2). However, LMB fed the diet containing 50% CP, 7.5% lipid and 14.9% starch) exhibited lower growth performance than LMB fed the diet containing 50% CP, 10% lipids and 9.2% starch. Moreover, even the 9.2% starch level in the 50% CP plus 10% lipid diet was not desirable for the LMB because their feed intake decreased after day 28 of the trial. Our findings also help to understand the cause of hepatic disorders in the liver of LMB fed the current commercial diets containing a high level of starch. Specifically, the enlargement of their liver and hepatosis due to excessive glycogen accumulation occurred in all the LMB fed the 40% CP diet (Figure 1) possibly because of high dietary starch levels (22-28%). However, hepatosis appeared to a lesser extent in LMB fed the 45% or 50% CP diet. Thus, we recommend that the diets of LMB contain < 9.2% starch (dry matter basis). These findings not only provide a much-needed basis for designing cost-effective and healthy diets for LMB but will also have important implications for understanding metabolic syndrome in humans who are susceptible to high dietary starch intake (Jobgen et al. 2006).

AST and ALT activities in serum are indicators of hepatic injury in animals (Wu, 2013), including many fish species (Li et al. 2014; Kimand and Lee, 2009). However, our results indicated that LMB fed 40% CP diets had lower AST activity than LMB fed 45% and 50% CP, even though the former had a clear symptom of glycogenic hepatopathy as shown by histological analysis (Fig IV-1). Li et al. (2014) suggested that a reduced activity of ALT and AST can occur when the hepatic concentrations of α-ketoacids are reduced by a deficiency of dietary AAs. Results of our current study indicate that a diet containing 40% CP does not meet the protein requirement of

juvenile LMB for AAs, which may be the main reason for low AST activities in serum. Nonetheless, inadequate intake of dietary protein impairs protein synthesis in animal tissues, including ALT and AST in the liver (Wu 2013b). As a result, the AST and ALT activities may not be valid biochemical markers for hepatopathy when fish have low intake of dietary protein or AAs. Like, ALT and AST, LDH and ALP activities in serum have been regarded as indicators of some liver diseases (Kotoh et al. 2011; Giannini et al. 2005). We observed that increasing the dietary CP level from 40% to 50% progressively decreased the activity of ALP in serum as well as LDH and ALP activities in the liver of LMB. Hepatic expression of these two enzymes may be down-regulated by dietary AAs possibly as a response to the concentration of glycogen and oxidative stress in the liver. Further studies are warranted to test this hypothesis.

In conclusion, results of this study indicate that 45% CP, 10% lipids, and < 9.2% starch in diets are satisfactory for the growth and health of juvenile LMB. Our work also identified that this fish cannot tolerate a high dietary starch level. The hepatosis that often occurs in LMB fed compound diets containing ≥ 9.2% starch results from hepatic glycogenosis or glycogenic hepatopathy, which is related to prolonged elevations of blood glucose concentrations to promote glycogen synthesis in the liver. The pale liver syndrome in LMB is not caused by hepatic accumulation of lipids. Dietary starch level should be kept below 9.2% to prevent hepatic dysfunction as well as improve growth performance and feed efficiency in LMB. Our findings may have important implications for understanding metabolic syndrome in humans who are susceptible to high dietary starch intake.

Table IV-1 Composition of experimental diets

Ingredient (%, DM basis)	P40/L7.5	P40/L10	P45/L7.5	P45/L10	P50/L7.5	P50/L10
Fish meal[1]	44.5	44.5	50.1	50.1	55.6	55.6
SPC[2]	11.2	11.2	12.6	12.6	14.0	14.0
Soybean oil[3]	0.60	0.60	0.58	0.58	0.57	0.57
Poultry fat [4]	0.67	3.17	0.67	3.17	0.67	3.17
Fish oil menhaden[5]	1.22	1.22	0.61	0.61	0.00	0.00
Dextrinized starch[6]	28.1	22.3	21.5	15.8	14.9	9.2
Vitamin premix[7]	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[8]	1.00	1.00	1.00	1.00	1.00	1.00
Cellulose[9]	10.57	13.87	10.8	14.0	11.12	14.32
CMC [10]	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.14	0.14	0.14	0.14	0.14	0.14
Analyzed content (g/100 g of dry mater in diet) *						
Crude protein (CP)	39.9	39.7	45.1	45.3	50.1	50.1
Crude lipids	7.52	10.2	7.57	10.1	7.54	10.0
Phosphorus	1.45	1.46	1.63	1.59	1.83	1.80
Energy (kJ/g)	16.6	16.6	16.7	16.7	16.6	16.6
CP/Energy (mg/kJ)	24.0	23.9	27.0	27.1	30.2	30.2

* The content of dry matter (DM) in the diets (as-fed basis) was 97.0%.

[1] Omega Fish Meal (Corporate Headquarters of Omega Protein, Houston, Texas).[2] PROFINE® Soy Protein Concentrate (Dupont, WI, USA).

[3] Nutrioli Pure Soybean Oil (Ragasa, N.L., Mexico).

[4] Chicken fat (Tyson Foods, Arkansas, USA).

[5] Fish oil (Paragon, Illinois, USA).

[6] Maltodextrin (Baolingbao Biology, Shangdong, China).

[7] Vitamin premix (g/kg): vitamin A, 2.31; vitamin D_3, 2.02; vitamin E, 20.00; vitamin K_3, 1.2; vitamin C, 30.00; vitamin B_5, 10.87; inositol, 15.00; niacin, 14.00; vitamin B_6, 3.04; vitamin B_2, 3.00; vitamin B_1, 3.26; biotin, 0.15; folic acid, 0.6; vitamin B_{12}, 0.02; Choline chloride, 135.00; cellulose, 894.53.

[8] Mineral premix (g/kg): NaCl, 363.88; $MgSO_4.7H_2O$, 586.67; $FeSO_4.7H_2O$, 22.22; $AlCl_3.6H_2O$, 0.67; KI, 0.67; $CuSO_4.5H_2O$, 2.22; $MnSO_4$, 4.67； $CoCl.6H_2O$, 0.86; $ZnSO_4.7H_2O$, 18.09; Na_2SeO_3, 0.06.

[9] Microcrystaline cellulose 102 (Blue Diamond Growers, California, USA).

[10] Sodium carboxy methyl cellulose (Pro Supply Outlet, California, USA).

Table IV-2 Growth performance of juvenile LMB fed diets containing different protein and lipid levels[1]

Diet		Body weight (BW, g/fish)					Weight gain over the initial BW (%)			
Protein (P, %)	Lipids (L, %)	Initial	Day 14	Day 28	Day 42	Day 56	Day 14	Day 28	Day 42	Day 56
40	7.5	18.4	24.6^{b}	33.7^{c}	40.6^{b}	44.3^{c}	34.0^{c}	83.3^{c}	121^{b}	141^{c}
40	10	18.3	25.4^{b}	35.7^{bc}	41.8^{b}	47.0^{bc}	38.9^{ab}	95.1^{bc}	128^{b}	157^{bc}
45	7.5	18.4	25.8^{ab}	35.4^{bc}	43.0^{b}	46.8^{bc}	39.9^{ab}	92.2^{bc}	133^{b}	154^{bc}
45	10	18.4	26.8^{a}	37.4^{ab}	43.7^{b}	49.2^{b}	45.7^{a}	103^{ab}	137^{b}	167^{b}
50	7.5	18.4	25.1^{b}	35.6^{bc}	40.7^{b}	45.7^{bc}	36.7^{bc}	93.9^{bc}	122^{b}	149^{bc}
50	10	18.3	26.2^{ab}	38.2^{a}	47.0^{a}	52.5^{a}	42.7^{ab}	108^{a}	156^{a}	187^{a}
Pool SEM		0.02	0.20	0.37	0.53	0.67	1.08	2.03	3.10	3.71
Main effects										
40		18.3	25.0^{B}	34.7^{B}	41.2^{B}	45.6^{B}	36.5^{B}	89.2^{B}	125^{B}	149^{B}
45		18.3	26.3^{A}	36.4^{A}	43.0^{A}	48.0^{A}	42.8^{A}	97.6^{A}	135^{A}	161^{A}
50		18.4	25.6^{AB}	36.9^{A}	43.8^{A}	49.1^{A}	39.7^{A}	101^{A}	139^{A}	168^{A}
	7.5	18.4	25.2^{B}	34.9^{B}	41.4^{B}	45.6^{B}	36.87^{B}	89.8^{B}	125^{B}	148^{B}
	10	18.4	26.1^{A}	37.1^{A}	43.9^{A}	49.6^{A}	42.45^{A}	102.2^{A}	141^{A}	170^{A}
Two-way ANOVA										
P		0.292	0.008	0.004	0.011	0.011	0.020	0.005	0.031	0.017
L		0.451	0.004	0.000	0.001	0.000	0.003	0.000	0.002	0.000
P × L		0.758	0.891	0.836	0.002	0.085	0.955	0.850	0.014	0.015

1 Values are means with pooled SEM for 4 tanks per dietary group.

a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table IV-3 Feed intake, feed conversion ratio, and protein efficiency ratio (PER) of juvenile largemouth bass fed diets containing different protein and lipid levels[1]

Diet		Feed intake (FI, g/fish)					Feed conversion ratio (FI/weight gain)					PER (weight gain/protein intake, g/g)				
Protein (P) %	Lipids (L) %	Days 0-14	Days 14-28	Days 28-42	Days 42-56	Days 0-56	Days 0-14	Days 14-28	Days 28-42	Days 42-56	Days 0-56	Days 0-14	Days 14-28	Days 28-42	Days 42-56	Days 0-56
40	7.5	8.80	10.4	8.87	8.11^{b}	36.2^{b}	1.44^{a}	1.16^{a}	1.24^{b}	2.30^{a}	1.40^{a}	1.75^{bc}	2.18^{ab}	2.05^{a}	1.09^{c}	1.79^{b}
40	10	8.81	10.1	9.53	7.90^{b}	36.3^{b}	1.26^{ab}	0.98^{ab}	1.46^{ab}	1.58^{c}	1.26^{bc}	2.01^{ab}	2.55^{a}	1.75^{a}	1.60^{a}	1.99^{a}
45	7.5	8.82	10.2	9.06	8.39^{b}	36.5^{b}	1.18^{b}	1.11^{a}	1.24^{b}	2.10^{ab}	1.30^{ab}	1.88^{abc}	2.01^{b}	1.82^{a}	1.07^{c}	1.73^{b}
45	10	8.67	10.3	9.67	9.15^{ab}	38.8^{a}	1.04^{b}	1.02^{ab}	1.41^{ab}	1.63^{c}	1.26^{bc}	2.16^{a}	2.22^{ab}	1.59^{a}	1.39^{ab}	1.76^{b}
50	7.5	8.43	9.90	8.87	8.50^{b}	35.7^{b}	1.27^{ab}	0.95^{ab}	1.67^{a}	1.81^{bc}	1.32^{ab}	1.60^{d}	2.13^{ab}	1.20^{b}	1.13^{c}	1.53^{c}
50	10	8.79	10.6	10.3	9.88^{a}	39.6^{a}	1.13^{b}	0.89^{b}	1.11^{b}	1.66^{c}	1.17^{c}	1.78^{bc}	2.27^{ab}	1.82^{a}	1.21^{bc}	1.73^{b}
Pool SEM		0.06	0.10	0.16	0.18	0.50	0.03	0.03	0.05	0.07	0.04	0.05	0.04	0.05	0.05	0.05
Main effect means																
40		8.81	10.3	9.55	8.01^{B}	36.7	1.35^{A}	1.07^{A}	1.35	1.93	1.35	1.88^{AB}	2.36	1.89^{A}	1.36	1.86^{A}
45		8.74	10.2	9.37	8.77^{A}	37.1	1.11^{B}	1.06^{A}	1.32	1.87	1.26	2.03^{A}	2.14	1.71^{AB}	1.20	1.78^{A}
50		8.61	10.3	9.59	9.19^{A}	37.7	1.20^{B}	0.92^{B}	1.39	1.73	1.22	1.69^{B}	2.25	1.51^{B}	1.25	1.62^{B}
	7.5	8.68	10.2	9.17^{B}	8.33^{B}	36.4^{B}	1.27^{A}	1.07^{A}	1.23	1.81^{A}	1.34^{A}	1.73^{B}	2.13^{B}	1.66^{B}	1.12^{B}	1.66^{B}
	10	8.76	10.3	9.84^{A}	8.98^{A}	37.9^{A}	1.13^{B}	0.96^{B}	1.19	1.42^{B}	1.21^{B}	2.03^{A}	2.37^{A}	1.80^{A}	1.43^{A}	1.82^{A}
Two-way ANOVA																
P		0.432	0.998	0.788	0.005	0.380	0.003	0.015	0.770	0.155	0.086	0.008	0.094	0.011	0.085	0.009
L		0.534	0.409	0.031	0.022	0.044	0.005	0.016	0.437	<0.001	0.031	0.005	0.012	0.039	0.001	0.034
P × L		0.246	0.090	0.137	0.061	0.093	0.939	0.563	0.999	0.038	0.040	0.780	0.360	0.986	0.524	0.177

1 Values are means with pooled SEM for 4 tanks per dietary group.

a-d: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table IV-3 The viscerosomatic index (VSI), hepatosomatic index (HSI), hepatocyte size, and intraperitoneal fat ratio (IPFR) of juvenile LMB fed diets containing different protein and lipid levels[1]

Protein (P, %)	Lipids (L, %)	VSI (%)	HSI (%)	Hepatocyte size (μm^2)	IPFR (%)	Survival %
P40	L7.5	9.54^{a}	4.18^{a}	382^{a}	1.47^{b}	91.5
P40	L10	8.93^{ab}	4.46^{a}	295^{b}	1.62^{b}	95.8
P45	L7.5	8.37^{b}	3.47^{b}	254^{c}	1.65^{b}	97.9
P45	L10	9.23^{a}	3.63^{b}	288^{b}	2.14^{a}	95.8
P50	L7.5	7.15^{c}	2.25^{c}	118^{d}	1.62^{b}	95.8
P50	L10	6.76^{c}	1.86^{d}	112^{d}	2.05^{a}	91.5
Pool SEM		0.23	0.20	6.73	0.07	1.59
Main effect means						
P40		9.24^{A}	4.32^{A}	339^{A}	1.55^{B}	93.6
P45		8.80^{B}	3.55^{B}	271^{B}	1.89^{A}	96.9
P50		6.95^{C}	2.06^{C}	115^{C}	1.83^{A}	93.7
	L7.5	8.35	3.30	251^{A}	1.58^{B}	95.1
	L10	8.31	3.32	232^{B}	1.94^{A}	94.4
Two-way ANOVA						
P		< 0.001	< 0.001	< 0.001	< 0.001	0.432
L		0.796	0.887	0.008	0.001	0.753
P × L		0.003	0.026	< 0.001	0.303	0.430

[1] Values are means with pooled SEM for 4 tanks (5 fish/tank) per dietary group.
a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table IV-4 Composition of the liver in juvenile LMB fed diets containing different protein and lipid levels[1]

Protein (P, %)	Lipids (L, %)	Nutrients in liver (mg/liver)				Nutrients in liver (g/100 g of wet weight)			
		Water	Protein	Lipids	Glycogen	Water	Protein	Lipids	Glycogen
P40	L7.5	1475^{b}	155^{b}	27.0^{b}	300^{a}	69.0	7.27^{b}	1.26^{c}	14.1^{a}
P40	L10	1676^{a}	170^{ab}	33.5^{a}	332^{a}	69.3	7.03^{b}	1.39^{bc}	13.7^{ab}
P45	L7.5	1220^{c}	165^{ab}	19.8^{c}	238^{b}	70.3	9.47^{a}	1.14^{c}	13.0^{ab}
P45	L10	1191^{c}	177^{a}	28.1^{b}	236^{b}	68.5	10.2^{a}	1.62^{b}	13.5^{ab}
P50	L7.5	760^{d}	114^{c}	21.3^{c}	141^{c}	69.9	10.5^{a}	1.96^{a}	12.9^{ab}
P50	L10	691^{e}	104^{c}	16.8^{c}	124^{c}	69.7	10.5^{a}	1.70^{ab}	12.5^{b}
Pooled S.E.M.		60.0	6.19	1.29	18.9	0.25	0.33	0.07	0.36
Main effect means									
P40		1573^{A}	163^{A}	30.3^{A}	316^{A}	69.2	7.11^{B}	1.27^{B}	14.9^{A}
P45		1201^{B}	171^{A}	24.0^{B}	237^{B}	68.9	10.0^{A}	1.32^{B}	13.5^{B}
P50		723^{C}	109^{B}	19.1^{C}	135^{C}	68.4	10.7^{A}	1.84^{A}	12.7^{B}
	L7.5	1149	145	22.7^{B}	226	69.1	9.10	1.42	13.5
	L10	1182	151	26.2^{A}	231	68.7	9.42	1.55	13.2
Two-way ANOVA									
P		< 0.001	< 0.001	< 0.001	< 0.001	0.714	< 0.001	< 0.001	0.006
L		0.701	0.181	0.011	0.358	0.550	0.349	0.147	0.243
P × L		0.000	0.062	0.001	0.001	0.100	0.512	0.008	0.984

1 Values are means with pooled SEM for 4 tanks per dietary group.

a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table IV-5 Composition of the whole body and skeletal muscle of juvenile LMB fed diets containing different protein and lipid levels [1]

Protein (P, %)	Lipids (L, %)	Nutrients in whole body (% of wet weight)						Nutrients in skeletal muscle (% of wet weight)			
		Water	Protein	Lipids	Ash	Phosphorus	Calcium	Water (%)	Protein (%)	Lipids (%)	Glycogen (mg/g)
Initial fish		76.5	17.6	1.62	4.23	0.73	1.08				
P40	L7.5	69.8	17.6	5.55^{bc}	5.43^{ab}	0.98^{a}	1.67^{a}	76.5	20.0	1.06^{ab}	0.62^{a}
P40	L10	68.7	18.0	6.07^{sb}	5.64^{s}	0.94^{ab}	1.59^{ab}	77.0	20.0	1.04^{ab}	0.68a
P45	L7.5	70.9	17.2	5.10^{c}	5.23^{ab}	0.89^{ab}	1.51^{ab}	76.9	19.6	1.02^{ab}	0.51^{b}
P45	L10	69.8	17.8	6.53^{s}	4.92^{ab}	0.87^{ab}	1.43^{ab}	77.2	19.0	1.13^{a}	0.63^{a}
P50	L7.5	70.8	18.0	5.01^{c}	5.22^{ab}	0.90^{ab}	1.56^{ab}	77.3	18.9	0.93^{b}	0.53^{b}
P50	L10	68.7	18.2	6.94^{s}	4.48^{b}	0.81^{b}	1.37^{b}	76.7	20.1	1.06^{ab}	0.50^{b}
Pooled SEM		0.29	0.17	0.19	0.12	0.02	0.03	0.16	0.22	0.02	0.03
Main effect means											
P40		69.2	17.8	5.81	5.54^{A}	0.96^{A}	1.64^{A}	76.8	19.7	1.06^{A}	0.65^{A}
P45		70.4	17.5	5.82	5.08^{B}	0.88^{B}	1.47^{B}	76.8	19.6	1.07^{A}	0.57^{B}
P50		69.8	18.1	5.98	4.85^{B}	0.86^{B}	1.47^{B}	76.9	20.0	0.99^{B}	0.51^{B}
	L7.5	70.5	17.6	5.22^{B}	5.29	0.93^{A}	1.56^{A}	77.0	19.5	1.00^{B}	0.55
	L10	69.0	18.0	6.51^{A}	5.01	0.87^{B}	1.37^{B}	76.8	20.0	1.08^{A}	0.60
Two-way ANOVA											
P		0.166	0.347	0.617	0.002	0.005	0.011	0.948	0.733	0.020	0.004
L		0.007	0.303	< 0.001	0.113	0.030	0.040	0.529	0.340	0.015	0.251
P × L		0.610	0.923	0.010	0.189	0.677	0.568	0.632	0.118	0.062	0.385

1 Values are means with pooled SEM for 4 tanks per dietary group.

a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table IV-6 Retention (or productive value, %) of dietary energy and nutrients in the body of juvenile LMB fed diets containing different protein and lipid levels.

		Retention of dietary energy and nutrients in the body (%)				
Protein (P, %)	Lipids (L, %)	Crude protein	Lipids	Energy	Phosphorus	Calcium
P40	L7.5	30.7^{c}	69.5^{b}	29.1^{d}	49.3^{a}	57.8^{a}
P40	L10	35.7^{a}	61.7^{c}	33.9^{c}	49.6^{a}	59.2^{a}
P45	L7.5	28.6^{d}	69.2^{b}	29.3^{d}	37.8^{c}	47.8^{b}
P45	L10	33.4^{b}	70.7^{b}	36.0^{b}	43.8^{b}	48.1^{b}
P50	L7.5	27.6^{d}	67.4^{b}	29.6^{d}	35.0^{c}	45.2^{bc}
P50	L10	33.5^{b}	78.2^{a}	40.2^{a}	37.6^{c}	42.6^{c}
Pooled SEM		0.73	1.31	1.04	1.44	1.40
Main effect means						
P40		33.2^{A}	65.5^{B}	31.5^{B}	49.4^{A}	58.5^{A}
P45		31.0^{B}	70.0^{A}	32.7^{B}	40.8^{B}	48.0^{B}
P50		30.6^{B}	72.8^{A}	34.9^{A}	36.3^{C}	43.9^{C}
	L7.5	29.0^{B}	68.6	29.3^{B}	40.6^{B}	50.3
	L10	34.2^{A}	70.2	36.7^{A}	43.7^{A}	49.9
Two-way ANOVA						
P		0.070	0.001	0.009	< 0.001	< 0.001
L		< 0.001	0.272	<0.001	0.014	0.783
P × L		0.871	<0.001	0.027	0.194	0.395

1 Values are means with pooled SEM for 4 tanks per dietary group.

a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table IV-7 Percentages (%) of the gains of nutritionally dispensable amino acids to their dietary intakes in juvenile largemouth bass fed diets containing different protein and lipid levels

Protein (P) %	Lipids (L) %	Ala	Asn	Asp	Cys	Glu	Gln	Gly	Pro	OH-Pro	Ser	Tyr
P40	L7.5	31.7^{a}	27.0^{c}	22.2^{c}	37.8^{b}	22.7^{d}	20.5^{c}	35.3^{c}	40.4^{b}	39.8^{b}	28.8^{c}	37.5^{a}
P40	L10	33.2^{a}	31.3^{b}	25.7^{b}	41.9^{a}	25.7^{bc}	23.2^{b}	41.2^{a}	46.7^{a}	46.1^{a}	31.7^{a}	37.1^{a}
P45	L7.5	29.4^{b}	35.0^{a}	29.1^{a}	37.2^{b}	26.5^{b}	23.5^{b}	33.5^{d}	37.4^{c}	35.6^{c}	30.5^{ab}	34.1^{b}
P45	L10	26.9^{c}	32.8^{b}	27.3^{ab}	36.5^{b}	25.1^{c}	22.3^{b}	38.7^{b}	42.1^{b}	40.3^{b}	27.2^{d}	31.3^{c}
P50	L7.5	28.0^{bc}	31.6^{b}	26.2^{b}	32.8^{c}	24.6^{c}	22.2^{b}	32.0^{d}	35.4^{c}	33.6^{c}	28.7^{c}	27.6^{d}
P50	L10	30.6^{b}	36.5^{a}	30.2^{a}	36.8^{b}	28.1^{a}	25.0^{a}	39.4^{b}	42.9^{b}	41.1^{b}	29.2^{bc}	31.7^{c}
Pool SEM		0.57	0.75	0.64	0.50	0.42	0.39	0.56	0.88	0.95	0.48	0.82
Main effect means												
P40		32.4^{A}	29.2^{B}	24.0^{B}	39.9^{A}	24.2^{B}	21.9^{B}	38.3^{A}	43.5^{A}	43.0^{A}	30.2	37.3^{A}
P45		28.1^{B}	33.9^{A}	28.2^{A}	36.9^{B}	25.9^{A}	22.9^{AB}	36.1^{B}	39.7^{B}	37.9^{B}	28.9	32.7^{B}
P50		29.3^{B}	34.0^{A}	28.2^{A}	34.8^{C}	26.3^{A}	23.8^{A}	35.7^{B}	39.1^{B}	37.4^{B}	29.0	29.7^{C}
	L7.5	29.7	31.2^{B}	25.8^{B}	35.9^{B}	24.6^{B}	22.1^{B}	33.6^{B}	37.7^{B}	36.3^{B}	29.3	33.1
	L10	30.2	33.5^{A}	27.7^{A}	38.4^{A}	26.3^{A}	23.5^{A}	39.8^{A}	43.9^{A}	42.5^{A}	29.4	33.4
Two-way ANOVA												
P		0.010	<0.001	<0.001	0.007	0.046	0.020	<0.001	0.003	<0.001	< .001	<0.001
L		0.516	0.022	0.023	0.039	0.034	0.037	<0.001	<0.001	<0.001	0.963	0.759
P × L		0.046	0.011	0.011	0.018	0.025	0.026	<0.001	0.533	0.503	0.025	0.019

[1] Values are means with pooled SEM for 4 tanks per dietary group.
a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
A-C: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
OH-Pro , 4-hydroxyproline

Table IV-8 Percentages (%) of the gains of nutritionally indispensable amino acids to their dietary intakes in juvenile largemouth bass fed diets containing different protein and lipid levels

Lipids (L) %	Starch (S) %	Arg	His	Ile	Leu	Lys	Phe	Met	Thr	Trp	Val
P40	L7.5	27.6^{b}	32.0^{a}	26.9^{b}	28.3^{bc}	26.2^{b}	36.7^{b}	22.8^{c}	23.4^{c}	29.5^{b}	23.7^{b}
P40	L10	31.1^{a}	33.8^{a}	27.0^{b}	31.0^{a}	32.1^{a}	36.8^{b}	25.6^{b}	27.6^{b}	32.5^{a}	26.0^{a}
P45	L7.5	24.9^{c}	27.3^{b}	26.6^{b}	26.8^{d}	23.0^{c}	30.7^{c}	20.6^{d}	28.2^{ab}	28.7^{b}	26.3^{a}
P45	L10	22.2^{d}	34.6^{a}	29.4^{a}	29.4^{b}	23.2^{c}	44.6^{a}	28.8^{a}	30.0^{a}	29.0^{b}	21.9^{c}
P50	L7.5	23.4^{cd}	25.0^{b}	23.7^{c}	25.4^{e}	21.0^{c}	25.4^{d}	20.4^{d}	24.8^{c}	25.3^{c}	23.4^{b}
P50	L10	24.2^{cd}	28.5^{b}	24.9^{c}	27.6^{cd}	27.8^{b}	38.9^{b}	20.3^{d}	27.6^{b}	28.8^{b}	25.3^{a}
Pool SEM		0.70	0.64	0.49	0.47	0.91	1.34	0.85	0.57	0.39	0.47
Main effect means											
P40		29.3^{A}	32.9^{A}	26.9^{A}	29.6^{A}	29.1^{A}	36.8^{A}	24.2^{A}	25.5^{B}	31.0^{A}	24.9
P45		23.5^{B}	31.0^{B}	28.0^{A}	28.1^{B}	23.1^{B}	37.6^{A}	24.7^{A}	29.1^{A}	28.9^{B}	24.1
P50		23.8^{B}	26.8^{C}	24.3^{B}	26.5^{C}	24.4^{B}	32.2^{B}	20.4^{B}	26.2^{B}	27.1^{C}	24.3
	L7.5	25.3	28.1^{B}	25.7	26.8^{B}	23.4^{B}	31.0^{B}	21.3^{B}	25.5^{B}	27.8^{B}	24.5
	L10	25.8	32.3^{A}	27.1	29.3^{A}	27.7^{A}	40.1^{A}	24.9^{A}	28.4^{A}	30.1^{A}	24.4
Two-way ANOVA											
P		<0.001	<0.001	0.001	0.008	0.001	<0.001	<0.001	0.003	0.005	0.468
L		0.499	<0.001	0.069	0.003	0.001	<0.001	<0.001	0.001	0.018	0.992
P × L		0.380	0.259	0.132	0.175	0.284	0.710	0.646	0.080	0.103	0.559

[1] Values are means with pooled SEM for 4 tanks per dietary group.
a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
A-C: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
OH-Pro, 4-hydroxyproline

Table IV-9 Concentrations (mM) of glucose in the serum of juvenile LMB at different time points after consuming diets containing different protein and lipid levels[1].

Protein (P, %)	Lipids (L, %)	2 h	4 h	8h	12 h	24 h
40	7.5	4.60	7.22^{a}	8.22^{a}	5.59^{c}	3.82^{ab}
40	10	4.57	7.17^{a}	6.57^{a}	8.16^{a}	4.67^{a}
45	7.5	4.22	6.19^{a}	8.25^{a}	5.53^{c}	3.46^{b}
45	10	4.63	7.86^{a}	6.71^{a}	8.01^{ab}	4.45^{ab}
50	7.5	4.71	6.08^{ab}	6.33^{a}	6.51^{bc}	4.37^{ab}
50	10	4.47	5.54^{b}	4.97^{b}	5.55^{c}	4.01^{ab}
Pool SEM		0.11	0.28	0.35	0.33	0.15
Main effect means						
40		4.58	7.20^{A}	7.40^{A}	6.88^{A}	4.25
45		4.43	7.03^{A}	7.48^{A}	6.77^{A}	3.96
50		4.59	5.81^{B}	5.56^{B}	6.03^{B}	4.19
	7.5	4.51	6.50	7.60^{A}	5.88^{B}	3.74^{B}
	10	4.55	6.86	6.08^{B}	7.24^{A}	4.38^{A}
Two-way ANOVA						
P		0.807	0.000	< 0.001	0.029	0.453
L		0.848	0.738	0.005	0.011	0.023
P × L		0.525	0.166	0.062	< 0.001	0.024

[1] Blood samples were obtained on day 56 of the experiment. Values are means with pooled SEM for 4 fish per dietary group at each time point.
a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table IV-10 Activities of aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) in the serum and liver of LMB after consuming diets containing different protein and lipid levels[1]

		Activity of enzymes in serum (U/L)				*Activities of enzymes in liver (U/g protein)*			
Protein (P) %	Lipids (L) %	AST	ALT	LDH	ALP	AST	ALT	LDH	ALP
P40	L7.5	43.5^{c}	15.5^{b}	152^{a}	21.3^{a}	107^{d}	30.8^{c}	85.6^{b}	550^{a}
P40	L10	43.8^{c}	15.8^{b}	155^{a}	20.4^{a}	131^{c}	33.7^{c}	93.6^{a}	484^{a}
P45	L7.5	50.8^{b}	15.2^{b}	149^{a}	17.2^{b}	140^{c}	40.4^{b}	66.5^{c}	326^{b}
P45	L10	55.4^{a}	18.2^{a}	154^{a}	15.4^{b}	163^{b}	45.0^{b}	63.4^{d}	303^{b}
P50	L7.5	55.6^{a}	15.2^{b}	148^{a}	10.9^{c}	157^{b}	58.5^{a}	34.5^{e}	273^{b}
P50	L10	53.5^{ab}	18.2^{a}	136^{b}	12.0^{c}	189^{a}	58.8^{a}	34.9^{e}	181^{c}
Pooled SEM		1.20	0.45	2.6	0.73	5.8	2.3	4.2	23.7
Main effect means									
P40		43.7^{B}	15.7	154^{A}	20.9^{A}	119^{C}	32.3^{C}	90^{A}	517^{A}
P45		53.1^{A}	16.7	152^{A}	16.3^{B}	152^{B}	42.7^{B}	65^{B}	315^{B}
P50		54.6^{A}	16.7	142^{B}	11.5^{C}	173^{A}	58.7^{A}	35^{C}	227^{C}
	L7.5	50.0	15.3^{B}	150	16.5	135^{B}	43.2	62.2	383^{A}
	L10	50.9	17.4^{A}	148	15.9	161^{A}	45.8	64.0	323^{B}
Two-way ANOVA									
P		0.003	0.664	0.008	<0.001	0.002	< 0.001	< 0.001	<0.001
L		0.710	0.003	0.806	0.451	0.023	0.522	0.694	0.041
P × L		0.501	0.552	0.263	0.207	0.941	0.905	0.582	0.062

[1] Values are means with pooled SEM 6 fish per dietary group.

a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

A-C: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Figure IV-1 Representative pictures of the whole fish body, liver, intraperitoneal adipose tissue in LMB fed diets containing different protein and lipid levels

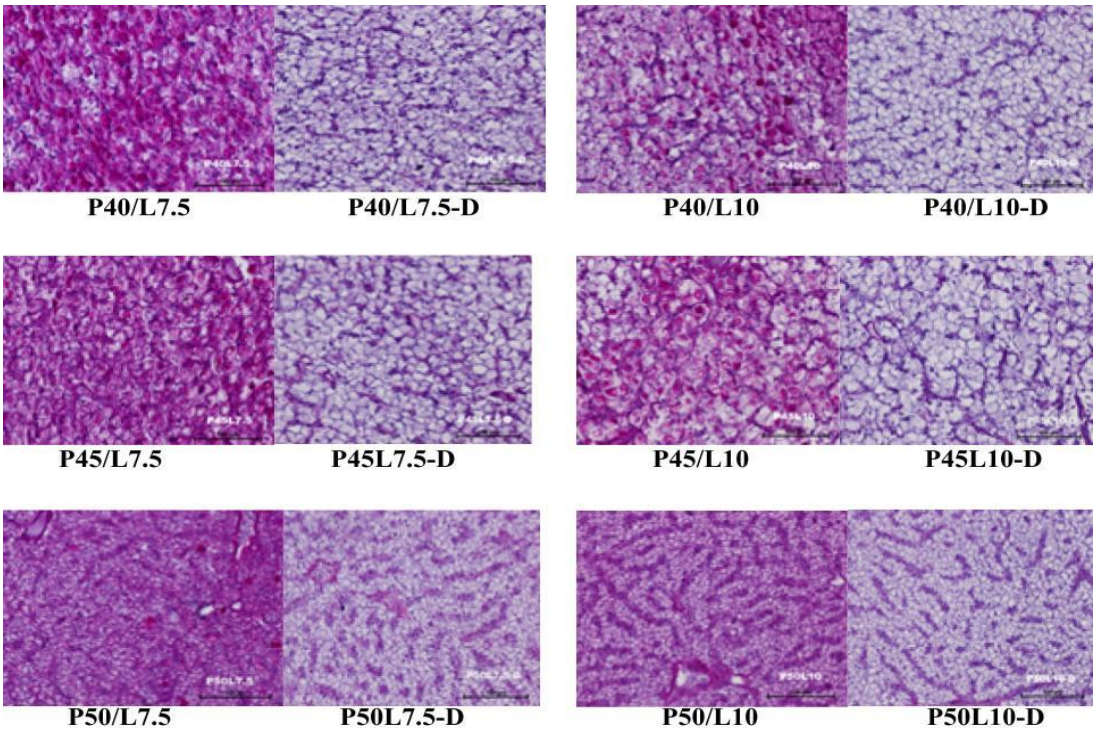


Figure IV-2 Hepatic histology of juvenile LMB fed diets containing different starch and lipid levels. The PAS stain (without diastase): showing the red staining of the hepatocyte cytoplasm. D refers to the PAS stain with diastase (to break down glycogen): showing a marked decrease in the bright red staining (or the amount of glycogen) of the hepatocyte cytoplasm. P, dietary crude protein level (%); L, dietary lipid level (%)

CHAPTER V

EFFECTS OF DIETARY STARCH AND LIPID LEVELS ON THE PROTEIN RETENTION AND GROWTH OF LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*)

Abstract Protein accretion in some fish species is affected by dietary lipids, starch and their interactions, but this aspect of nutrition is largely unknown in largemouth bass (LMB, a carnivorous fish). Therefore, we designed six experimental diets with three starch levels (5%, 10%, and 15%; dry matter basis) and two lipid levels (10% and 12.5%; dry matter basis) to evaluate the effects of dietary starch and lipid levels on the protein retention, growth, feed utilization, and liver histology of LMB. There were three tanks (18 fish per tank, ~4.85 g per fish) per dietary treatment group and the trial lasted for 8 weeks. Fish were fed to apparent satiation twice daily. Results indicated that increasing the dietary starch level from 5% to 15% reduced ($P < 0.05$) feed intake (-9.0%, -15% and -14% on days 14-28, 28-42, and 42-56, respectively) and weight gains (-4.4% and -6.5% on days 42 and 56, respectively) of LMB. Increasing the dietary lipid level from 10% to 12.5% reduced ($P < 0.05$) feed intake (-9.7%, -11.7% and -11.9% on days 14-28, 28-42; and 42-56, respectively), weight gains (-4.2%, -5.9% and -6.9% on days 28, 42 and 56, respectively), and survival rate (by a 5.6% unit) of LMB. The retention of dietary protein and some amino acids in the body was affected by dietary starch or lipid levels and their interactions. The viscerosomatic index (VSI), hepatosomatic index (HSI), and intraperitoneal fat ratio (IPFR) increased with increasing the dietary starch level from 5% to 15%. Compared with 10% lipids, 12.5% lipids in diets increased IPFR but had no effect on VSI or HSI. The concentrations of glucose in serum increased with increasing the dietary starch level from 5% to 15% at 4 to 24 h after feeding. Compared with 10%-lipid diets, concentrations of serum glucose in fish fed diets with 12.5% lipids

were lower at 2-8 h after feeding, but higher at 24 h after feeding. Compared with a 5%-starch diet, fish fed a diet with 10%- or 15%-starch exhibited an enlarged and pale liver with excessive glycogen. Based on these findings, we recommend dietary lipid and starch levels to be 10% and < 10%, respectively, for juvenile LMB to maximize the retention of dietary protein in their bodies.

Introduction

Fish have particularly high requirements for dietary protein because AAs are their major metabolic fuels (Jia et al. 2017). In order to reduce protein requirement and feed costs, it is important to improve AA utilization for protein synthesis rather than as an energy source (Mohanta et al. 2008). In swine and poultry nutrition, the non-productive use of ingested protein is generally reduced considerably when a proper amount of starch or lipids is added to diets (Wu 2018). As a major non-protein energy source in fish diets, starch is the least expensive form of dietary energy, and the protein-sparing effect of dietary starch has been reported in some fish species (Jafri 1995; Wang et al. 2005; VásquezTorres and Arias-Castellanos 2013). The inclusion of starch can also improve the physical properties of extrusion and steam pellet feeds (Wilson 1994). However, for carnivorous fish, dietary lipids are regarded as better providers of energy than digestible carbohydrate, because utilization of glucose by their tissues is limited (Cowey and Sargent 1977; Jia et al. 2017). Moreover, appropriate levels of dietary lipids or starch should be carefully evaluated and determined for fish in order to enhance their growth, health, and product quality. Excessive lipids and starch in diets could lead to growth restriction, hepatopathy, poor immune function, and excess fat accumulation in fish (Han et al. 2014a; Li et al. 2015; Wang et al. 2016; Yang et al. 2018; Zhou et al., 2020).

LMB is a carnivorous fish, which is cultured in North America **(**Durborow 2019) and many other countries [including China (Tidwell et al. 2019; Wang et al., 2015)] worldwide as an

aquaculture species due to its high market value. In Chapter II and III, we found that LMB extensively oxidize AAs as metabolic fuels but have a limited ability to utilize glucose or fatty acids for ATP production. The requirement of this fish for dietary protein is about 45% (dry matter basis; Chapter III and IV). However, little is known about the requirements of LMB for dietary lipids and starch or their interactions. Therefore, we conducted this study to evaluate the effects of dietary starch (5%, 10% and 15%) and lipids (10% and 12.5%) levels (dry matter basis) on the growth, feed utilization, glucose metabolism, and liver histology of LMB.

Materials and Methods

Experimental diets

Six experimental diets were formulated to contain three starch levels (5%, 10%, and 15%; dry matter basis) and two crude lipid levels (10% and 12.5%; dry matter basis) with a fixed level of 45% protein. The ingredients and proximate composition of diets are shown in Table V-1. The main protein sources used in this study are fishmeal and soybean protein concentrate with defined composition of AAs, carbohydrate and lipids (Li and Wu 2020). The main lipid sources were fish oil, poultry fat, and soybean oil. All solid ingredients were thoroughly mixed using a mixer and thereafter, fish oil, soybean oil, and water were added to the mixture to form a moist dough. The experimental diets were produced by a screw extruder (Big Bite Meat Grinder, West Chester, OH) and oven-dried at 50 ℃ until dry matter content was 97.0%. All feeds were kept at – 20 ℃.

Experimental animals

Juvenile LMB were obtained from a commercial fish farm (Bait Barn, Bryan, TX, USA), and maintained in a recirculating water system. Prior to starting the experiments, LMB were acclimated to the experimental condition and maintained on a commercial diet (AquaMax® Grower 400, Purina, MO) for 2 weeks. The recirculating experimental system for the culture of fish consisted

of 18 tanks, with three tanks for each dietary treatment. Each tank contained 55 L of deionized water (26.0 ± 0.01 ^{o}C; mean ± SEM, n = 56). At the beginning of this trial, 18 fish with a uniform body size [initial mean body weight, about 4.8 g (ranging from 4.7 to 4.9 g) per fish] were randomly distributed into each tank. Air was supplied to the water through air stones connected to air pumps, with its salinity being maintained at 3-5 ppt. The photoperiod of the housing facility was maintained for 14 h per day, with lights being turned off between 10:00 PM and 8:00 AM. Water quality parameters [pH 6.90 ± 0.03, NH_4^+ (0.48 ± 0.02 mg/L), nitrite (0.25 ± 0.01 mg/L), nitrate (11.1 ± 0.07 ppm), and dissolved O_2 (8.0 ± 0.05 ppm); mean ± SEM, n = 56] were monitored daily and remained within acceptable limits. Fish were hand-fed with experimental diets to apparent satiation twice daily at 09:00 and 16:00. Total feed consumption was recorded every day. The amount of the unconsumed diet supplied to each tank was collected to determine the weight of feed based on the number of pellets. We determined that the loss of provided pellet feed into the water was 10%, and therefore, the true feed consumption by fish was calculated as the amount of provided feed x 0.9. Total fish weight in each tank was recorded every two weeks to minimize handling and stress. Fish were weighed after a 24-h period of food deprivation. Almost 100% water in tanks was replaced gradually every day by adding fresh water into a central reservoir tank at the rate of ~ 4 L/min for 5 h to make sure the quality of water is optimal. The feeding trial lasted for 56 days.

Sample collection

At the beginning of the trial, 30 fish were euthanized with 140 ppm MS-222 (neutralized by an appropriate amount of $NaHCO_3$) for the analysis of whole-body composition. At the end of the experiment, blood (0.5 ml) was collected from the caudal vein of conscious fish (6 fish/tank) with the use of a hypodermic syringe at various time points after feeding. After blood collection, all fish

were euthanized as described previously, with four fish being randomly selected from each tank for the analyses of whole-body composition and with 4 fish being randomly selected from each tank to obtain viscera organs [including the stomach and intestine (without luminal contents), as well as liver, pancreas, and spleen)], peritoneal adipose tissue, and skeletal muscle. Blood samples were immediately centrifuged (2 min at 8000 g) at 4 ℃. The supernatant fluid (serum) was obtained and stored at -80 ℃ until analyzed.

Biochemical analyses

Crude protein (N × 6.25) was determined by the combustion method, and minerals were determined by optical spectrometry in Servi-Tech laboratories (Amarillo, TX, USA). Moisture was determined by drying at 105 ℃ in an oven to a constant weight. Lipids were extracted from the samples with chloroform/methanol (2:1 v/v) according to the method of Folch et al. (1957). Glucose in neutralized samples was determined enzymatically using a fluorometric method involving hexokinase and glucose-6-phosphate dehydrogenase (Fu et al. 2005), and lactate was analyzed by using lactate dehydrogenase (Wu et al. 1995). Gross energy contents values of diets were calculated based on 22.6 kJ/g, 39.3 kJ/g and 17.2 kJ/g for crude protein, crude lipid, and glycogen/starch, respectively. Amino acids in the diets and the bodies of fish were analyzed, as described by Li and Wu (2020).

Syntheses of glycogen and lipids from glucose in the liver and skeletal muscle

Each fresh tissue (20-50 mg) was placed into a tube with 1 ml KHB buffer containing 5 mM glucose and [U-^{14}C]glucose (150 dpm/nmol). Each tube was incubated at 26 ℃ for 2 h after the incubation medium was gassed with 95% O_2/5% CO_2 for 20 sec. At the end of the 2-h incubation, all tubes were centrifuged for 3 min at 600 g and the tissue was obtained for glycogen analysis, as described by Jobgen et al. (2009) with modifications. Briefly, tissue was washed with ice-cold

PBS four times. Thereafter, 0.3 ml of 30% KOH was added to the tissue. The solution was boiled for 5 min in a water bath for solubilization, followed by addition of 0.1 ml of 2% Na_2SO_4 and 0.9 ml of 100% alcohol. Then, all the tubes were kept as 25 °C for overnight. The pellet (glycogen-sodium sulphate) was obtained by centrifugation at 600 g for 10 min, and the supernatant fluid was saved for the extraction of glycogen associated with protein. The glycogen pellet was washed with 2 ml of 65% alcohol twice. After the alcohol was evaporated in a water bath at 70 °C, the precipitate was dissolved in 0.1 ml of 1 M H_2SO_4. The solution was transferred to a counting vial with 5 ml of scintillation cocktail and ^{14}C radioactivity was measured by a liquid scintillation counter (Zhang et al. 2019). The supernatant fluid from the glycogen-sodium sulfate precipitation step above was neutralized with 6 M HCl (about 265 ul), followed by addition of 1 ml of 1.5 M $HClO_4$ and centrifugation (10 min at 600 g rpm). The resultant pellet was washed with 2 ml of 1.5 M $HClO_4$ three times and dissolved in 0.3 ml of 30% KOH. An aliquot (0.2 ml) of this solution was mixed with 5 ml of scintillation cocktail for the measurement of ^{14}C radioactivity in a liquid scintillation counter (Zhang et al. 2019).

^{14}C-Lipids was extracted from tissue with the use of isopropylalcohol: heptane: 1N H_2SO_4 (40:10:1; extraction solution), as described by Wu et al. (1991). Briefly, each tissue was homogenized with 5 ml of extraction solution. The homogenate and incubation medium were mixed, and vortex for 1 min. After 5 min, 3 ml Heptane and 3 ml H_2O was added to each tube. All the tubules were vortexed for 2 min. After 5 min, the upper layer (heptane layer) was collected, and heptane was dried down to less than 0.5 ml at 65 °C. Finally, 5 ml of scintillation cocktail was added to the tube for the measurement of ^{14}C radioactivity by a liquid scintillation counter.

Histological analysis of the liver

A portion (500 mg) of the liver was fixed with 4% paraformaldehyde (buffered to pH 7.2) for 24 h. The samples were rinsed, dehydrated, and embedded in paraffin for histological analysis at the Veterinary Medicine & Biomedical Sciences Histology Laboratory of Texas A&M University. Each sample was cut into two of 6 μm transverse sections with a rotary microtome. The sections were stained with the periodic acid-Schiff (PAS) solution as described by Fu and Campbell-Thompson (2017). One section was digested by α-amylase, and the other was treated with the vehicle buffer, and both were stained with the PAS solution. The PAS solution containing diastase (an enzyme that digests glycogen) was also used to stain the tissue for differentiating glycogen from other PAS-positive elements in tissue. For each treatment group, the size of hepatocytes was calculated as the area of cells in histology slides (10 cells for each liver sample). The hepatocyte images in the histology slides were evaluated by using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axiocam HR digital camera, the cell area was measured by Axiovision 4.3 software (Carl Zeiss).

Calculation and statistical analysis

Growth performance, feed utilization and morphometrical parameters were calculated as described in Chapter IV. All data was tested for homogeneity (the Levene's test) and normal distribution (the Kolmogorov-Smirnov test). When the tests were statistically significant, the data were log transformed before analysis. A 2×3 factorial analysis of variance (ANOVA) was used to analyze the main effects (lipids and starch) and their interactions. The tank for fish rearing was the experimental unit for data analyses. Differences among treatment groups were analyzed by the Student-Newman-Keuls multiple comparison test. $P < 0.05$ was taken to indicate statistical

significance. All statistical analyses were performed using the SPSS package (version 18.0, Chicago, IL).

Results

Absoulte feed intake (AFI), growth performance, feed efficiency, and survival of LMB

Data on the AFI [as done by other researchers (Borges et al. 2009; Rueda-Jasso et al. 2004)], growth performance, and feed efficiency of LMB are summarized in Tables V-2 and 3. Between days 0 and 14 of the trial, increasing the dietary starch level from 5 to 15% or the dietary lipid level from 10 to 12.5% did not affect ($P > 0.05$) the feed intake or weight gains of LMB. Between days 14 and 28 of the trial, increasing the dietary starch level from 5 to 15% reduced ($P < 0.05$) the feed intake of LMB by 9.0% but had no effect ($P > 0.05$) on their weight gain; increasing the dietary lipid level from 10 to 12.5% reduced ($P < 0.05$) the feed intake of LMB by 9.7% and their weigh gains by 4.2%. After day 28 of the trial, increasing the dietary starch level from 5 to 15% reduced ($P < 0.05$) feed intake (-15% and -14% on days 28-42 and 42-56, respectively) and weight gains (-4.4% and -6.5% on days 42 and 56, respectively) of LMB, whereas increasing the dietary lipid level from 10 to 12.5% reduced ($P < 0.05$) feed intake (-11.7% and -11.9% on days 28-42 and 42-56, respectively) and weight gains (-5.9% and -6.9% on days 28-42 and 42-56, respectively) of the fish. During the entire 8-week experiment, the relative feed intakes (% of body weight per day) of the LMB in the 5%, 10% and 15% starch groups were 2.70 ± 0.13, 2.58 ± 0.12, and 2.56 ± 0.12 (means ± SEM, n = 6 tanks), respectively ($P > 0.05$), whereas the values for fish in the 10% and 12.5% lipid groups were 2.66 ± 0.09 and 2.56 ± 0.08 (means ± SEM, n = 9 tanks), respectively ($P > 0.05$). Neither feed conversion ratio (feed intake/weight gain) nor protein efficiency ratio (weight gain/protein intake) was affected ($P > 0.05$) by dietary starch (5 to 15%) or lipid (10 to 12.5%) levels during the entire experimental period. There were no interactions (*P*

> 0.05) between dietary starch and lipid levels in affecting the food intake or weight gain of LMB. Thus, overall, juvenile LMB consumed less feed and grew more slowly when fed a 45%-protein diet containing either ≥ 10% starch or 12.5% lipids. The absolute weight gain of fish did not appear to be closely related to the percentages of dietary AAs retained in the body (Tables V-5 and V-6).

The rates of survival of LMB were 93% to 99%, depending on dietary groups (Table V-5). Increasing the dietary starch level from 5 to 15% did not affect ($P > 0.05$) the survival of LMB. In contrast, increasing the dietary lipid level from 10 to 12.5% reduced ($P < 0.05$) the survival rate of LMB by a 5.6% unit. There were no interactions ($P > 0.05$) between dietary starch and lipid levels in affecting the survival of the fish.

Retention (or productive value, %) of dietary energy, protein, lipids, phosphorus, calcium and amino aicds in the body of LMB

During the entire 56-day trial, 35% to 41% of dietary energy, 29% to 33% of dietary protein, 35-40% of dietary phosphorus, and 46-50% of dietary calcium were retained in the body of LMB, whereas the ratios of lipids gained in the body to their dietary intake (productive value) were 48% to 71% (Table V-4). Increasing the dietary starch level from 5 to 15% or the dietary lipid level from 10 to 12.5% did not affect ($P > 0.05$) the retention of dietary phosphorus and calcium in the body of LMB. In contrast, increasing the dietary starch level from 5 to 15% had no effect ($P > 0.05$) on the productive value of dietary energy in the body of LMB, reduced ($P < 0.05$) the retention of dietary protein by 3.2%, and increased ($P < 0.001$) the productive value of dietary lipids by 26.7%. Increasing the dietary lipid level from 10 to 12.5% reduced ($P < 0.05$) the productive value of dietary energy, protein, and lipids by 8.3%, 2.2%, and 17.8%, respectively. There were interactions between dietary starch and lipid levels in affecting the productive of dietary energy ($P < 0.001$), protein ($P < 0.001$), lipids ($P < 0.05$), phosphorus ($P < 0.001$) and

calcium (P = 0.020) in the body of LMB. Specifically, the retention of dietary energy, protein and phosphorus in the body of LMB fed the 10% starch and 10% lipid was the highest ($P < 0.05$) among all the dietary groups, whereas the productive value of dietary lipids in the body of LMB fed the 15% starch and 10% lipid diet was the highest ($P < 0.05$) among all the dietary groups. The retention of dietary phosphorus in LMB fed the diet containing 10% starch and 10% lipids was the highest ($P < 0.05$) among all the dietary groups, whereas the retention of dietary calcium in LMB fed the diet containing 10% starch and 10% lipid diet, the 5% starch and 10% lipid diet, and the 15% starch and 12.5% lipid diet was higher ($P < 0.05$) than other groups of the fish. Data on the ratios of amino acids gained in the body to their dietary intakes are summarized in Table V-5 (for nutritionally dispensable amino acids) and Table V-6 (for nutritionally indispensable amino acids). There were interactions between dietary starch and lipid levels in affecting the retention of most dietary amino acids ($P < 0.05$). Increasing the dietary starch level from 5 to 10% increased the ratios of aspartate, glutamate, cysteine, proline, glycine, and valine gained in the body to their dietary intake, whereas increasing the dietary lipid level from 10 to 12.5% did not affect the retention of dietary amino acids ($P > 0.05$).

Relative weights of all viscera organs, liver, and intraperitoneal adipose tissue of LMB

Figure V-1 shows the pictures of the body, liver and intraperitoneal adipose tissue of LMB fed diets containing different starch and lipid levels. Data on the VSI, HSI, IPF, and hepatocyte size of juvenile LMB fed different diets are summarized in Table V-7. Viscera organs, liver, and IPF represented 7.3-8.5%, 1.1-1.9%, and 2.2-3.0% of the whole-body weight, respectively. All of the three variables increased ($P < 0.05$) with increasing the dietary starch level from 5 to 15%. Increasing the dietary lipid level from 10 to 12.5% increased ($P < 0.001$) IPF by 16.2% but had no effect ($P > 0.05$) on VSI or HSI. There were no interactions ($P > 0.05$) between dietary starch and

lipid levels in affecting the VSI, HSI or IPF of LMB. Increasing the dietary starch level from 5 to 15% augmented ($P < 0.001$) the hepatocyte size in the liver by 75.9%, but increasing the dietary lipid level from 10 to 12.5% had no effect. There were interactions ($P < 0.05$) between dietary starch and lipid levels in affecting the hepatocyte size of LMB, in that the hepatocyte size in the liver of LMB fed the 15 starch and 10% lipid diet was the largest ($P < 0.05$) among all the dietary groups.

Composition of macronutrients in the body of LMB

At the end of the 56-day trial, the content of water, protein, lipids, phosphorus, and calcium in the body of LMB costituted 70-72%, 17%, 6.3-7.6%, 0.75%, and 1.2%, respectively, whereas the content of water, protein, and lipids in the skeletal muscle of LMB was 77%, 20%, and 1.2%, respectively (Table V-8). Increasing the dietary starch level from 5 to 15% did not affect ($P > 0.05$) the content of protein, phosphorus, and calcium in the body of LMB or the content of water and protein in their skeletal muscle, but increased the content of lipids in both the body (+20.5%; $P = 0.005$) and skeletal muscle (+14.3%; $P = 0.027$). Compared with the 5%-starch diet, the LMB fed the 10% starch diet had a 1.8% lower content of water in the body. Increasing the dietary lipid level from 10 to 12.5% had no effect ($P > 0.05$) the content of protein, phosphorus and calcium in the body or the content of water and lipids in skeletal muscle, increased ($P = 0.002$) the content of lipids in the body by 16.4%, and reduced ($P < 0.01$) the content of water in the body by 1.5% and the content of intramuscular protein by 1.5%. There were no interactions ($P > 0.05$) between dietary starch and lipid levels in affecting the content of water and lipids in the body of LMB or the content of water, protein and lipids in skeletal muscle. However, there were interactions ($P < 0.05$) between dietary starch and lipid levels in affecting the content of protein, phosphorus and calcium in the body. Specifically, the retention of dietary protein in the body of LMB fed the 10%

starch and 10% lipid diet and the 5% starch and 12.5% lipid diet was the highest ($P < 0.05$) among all the dietary groups, whereas the retention of dietary phosphorus and calcium in the body of LMB fed the 10% starch and 10% lipid diet, the 5% starch and 12.5% lipid diet, and the 15% starch and 12.5% lipid diet was the highest ($P < 0.05$) among all the dietary groups.

Composition of macronutrients in the liver of LMB

At the end of the 56-day trial, the content of water, protein, lipids, and glycogen in the body of LMB was about 73-77%, 16.9-17.7%, 1.6-2.8%, and 8-12%, respectively (Table V-9). Increasing the dietary starch level from 5 to 15% enhanced ($P < 0.001$) the content of water (+3.7%) and glycogen (+41%) in the liver of LMB, but decreased ($P < 0.001$) the content of protein (-20.8%) and lipids (-39.1%). Increasing the dietary lipid level from 10 to 12.5% also decreased the content of protein in the liver (-10.5%; $P = 0.002$) and lipids (-9.9%; $P = 0.020$), but did not affect ($P > 0.05$) the content of water or glycogen in the liver. There were interactions ($P < 0.05$) between dietary starch and lipid levels in affecting the content of water in the liver of LMB. Specifically, the content of water in the liver of LMB fed the 5% starch and 12.5% lipid diet was the highest ($P < 0.05$) among all the dietary groups.

Syntheses of glycogen and lipids from glucose in the liver and skeletal muscle

To assess the metabolic rates of glycogen and lipid syntheses from glucose, livers and skeletal muscle from the six different groups of LMB were incubated with the same extracellular concentration of glucose (5 mM). The rates of glycogen synthesis from glucose in the liver of LMB were about three times those for fatty acid synthesis (Table V-10). Compared with the liver, the skeletal muscle of LMB had a very low activity to synthesize glycogen and fatty acids from glucose. The rates of glycogen synthesis in the liver or skeletal muscle did not differ ($P > 0.05$) between LMB fed 5% and 15% starch diets. Likewise, the rates of fatty acid synthesis from glucose

did not differ ($P > 0.05$) among LMB fed the 5%, 10% and 15% starch diets. Of interest, there was a tissue-specific effect of dietary lipid levels on the rates of fatty acid and glycogen syntheses in the liver and skeletal muscle. Specifically, increasing the dietary lipid level from 10 to 12.5% did not affect ($P > 0.05$) the rates of fatty acid synthesis from glucose in the liver but increased ($P < 0.05$) those in skeletal muscle by 19.7%. In contrast, increasing the dietary lipid level from 10 to 12.5% did not affect ($P > 0.05$) the rates of glycogen synthesis from glucose in skeletal muscle but decreased ($P < 0.05$) those in the liver by 12.4%. There were no interactions ($P > 0.05$) between dietary starch and lipid levels in affecting fatty acid and glycogen syntheses in the liver or skeletal muscle of LMB.

Concentrations of glucose and lactate in the serum of LMB

Concentrations of glucose in the serum of LMB were 4.64, 3.92, 3.41, 1.82 and 2.11 mM (pooled SEM = 0.12; n = 18 tanks per time point) at 2, 4, 8, 12, and 24 h after feeding. The concentrations of glucose in serum decreased ($P < 0.001$) progressively between 2 and 12 h after feeding and remained low at 24 h after feeding, with no difference ($P > 0.05$) between 12 and 24 h after feeding. Increasing the dietary starch level from 5 to 15% elevated ($P < 0.001$) the concentrations of glucose in serum at 2 to 24 h after feeding. In contrast, the effect of dietary lipids on the concentrations of glucose in serum was time-dependent. Specifically, increasing the dietary lipid level from 10 to 12.5% decreased ($P < 0.05$) the concentrations of glucose in serum by 13.7% to 17.6% at 2 to 8 h after feeding, had no effect ($P > 0.05$) at 12 h after feeding, and increased ($P < 0.05$) those by 23.1% at 24 h after feeding. At 4 h after feeding, there were interactions ($P = 0.006$) between dietary starch and lipid levels in affecting the concentrations of glucose in the serum of LMB. Specifically, the concentrations of glucose in serum was higher ($P < 0.05$) when the 45% protein diet contained 15% starch and 10% lipids, compared with the 5% starch and 10%

lipid diet, the 10% starch and 10% lipid diet, and the 5-15% starch and 12.5% lipid diets. At other time points, there were no significant interactions between dietary starch and lipid levels in affecting the concentrations of glucose in serum.

Concentrations of lactate in the serum of LMB were 2.73, 2.06, 2.14, 2.18 and 2.46 mM (pooled SEM = 0.09; n = 18 tanks per time point) at 2, 4, 8, 12, and 24 h after feeding. The concentrations of lactate in serum decreased ($P < 0.001$) between 2 and 4 h after feeding, remained at reduced values through 12 h after feeding, and increased ($P < 0.05$) at 24 h after feeding, compared with 4 to 12 h after feeding. Increasing the dietary starch level from 5 to 15% enhanced ($P < 0.001$) the concentrations of lactate in serum at 2 to 24 h after feeding. In contrast, increasing the dietary lipid level from 10 to 12.5% did not affect ($P > 0.05$) the concentrations of lactate in serum at 2 to 24 h after feeding. At 4 to 12 h after feeding, there were interactions ($P < 0.05$) between dietary starch and lipid levels in affecting the concentrations of lactate in the serum of LMB. Specifically, the 4-h concentrations of lactate in the serum of LMB fed the 15% starch and 10% lipid diet was the highest ($P < 0.05$) among all the dietary groups, whereas the 8- and 12-h concentrations of lactate in the serum of LMB fed the 15% starch and 10% lipid diet, 10-15% starch and 12.5% lipid diets were higher ($P < 0.05$) than those in the 5-10% starch and 10% lipid diets and the 5% starch and 12.5% lipid diet. At 2 and 24 h after feeding, there were not significant interactions ($P > 0.05$) between dietary starch and lipid levels in affecting the concentrations of lactate in serum.

Liver histology

Histological analyses revealed that, compared with LMB fed the 5% starch diet, LMB fed 10-15% starch diets exhibited an enlarged and pale liver, as well as enlarged hepatocytes with

excessive glycogen in the cytosol, as indicated by the PAS stain (Fig V-2). These histologic findings are characteristics of glycogenic hepatopathy (hepatic glycogenosis).

Discussion

Results of this study indicated that juvenile LMB adapted well to our culture system on the basis of the rates of their growth and survival. The lean tissue gain in fish is mainly determined by the deposition of protein in the body (Dumas et al. 2007). We noted a few reports that LMB could achieve a rate of up to 40% of dietary nitrogen retention in the body (Li et al. 2018, 2019; Ding et al. 2019). Our lower values (29-33%) for this variable are similar to or better than those for similar size LMB that were fed similar diets, such as 22-30% (Portz et al. 2001), 25-30% (Subhadra et al. 2006), 26-28% (Tidwell et al. 2007), 30% (Gong et al. 2019; Song et al. 2018), 25% (Cai et al. 2020), 17-22% (Guo et al. 2020), ~ 20% (Zhong et al. 2020), and 25-30% (Zhou et al. 2014). The average retention of dietary nitrogen in juvenile LMB is ~ 30% (Tidwell et al. 2019), which is lower than the value of 55-65% in poultry (Rehman et al. 2018) and 45-70% for growing pigs depending on age (Wu et al. 2014). Fry et al. (2018) indicated that the average rates of the retention of dietary nitrogen as edible protein in aquatic species and chickens were 19% and 37%, respectively (Fry et al. 2018), and the value is 41% for pigs (Wu et al. 2014). Dietary amino acids that are not retained in the body are either oxidized or excreted into the environment. A number of environmental factors, such as temperature, oxygen concentration, salinity, density and photoperiod, can influence the growth rate of farmed fish (Chen et al. 2015; Matthias et al. 2018; Ren et al. 2018). In the current study, a relatively high density (18 fish/55 L of water) may be a reason for the relatively lower rate of growth. Moreover, differences in genotypes and the culture systems may also influence the growth rate of fish (Gjedrem 2000; Pierce et al. 2008). Of note, fish generally have 1.5- to 2-fold greater requirements for dietary protein (30% to 55%) than

livestock species and poultry (12% to 20%), and use more amino acids as metabolic fuels (Wu 2018). In support of this view, we recently reported that tissues (the proximal intestine, liver, skeletal muscle, and kidneys) of hybrid-striped bass (Jia et al. 2017) and LMB (Li and Wu 2019; Li et al. 2020) prefer to use glutamate, glutamine and aspartate to provide ATP.

Appropriate dietary starch and lipid levels could improve the protein retention and growth performance of fish, but the nutritional effects likely vary among different species (NRC 2011). Despite the wide use of LMB in aquaculture, little is known about their dietary requirements for starch and lipids for maximum protein accretion. We found that the retention of dietary nitrogen in the LMB, as well as the ratios of some amino acids gained in the body to their dietary intakes, increased with increasing the dietary starch level from 5 to 10% (Tables V-5 and 6), and the growth of LMB decreased with increasing the dietary starch level from 5 to 15% or the dietary lipid level from 10 to 12.5% (Table V-2). Interestingly, the ratio of glycine gained in the body to its dietary intake was about 43%, which was greater than that for any of the other amino acids (Table V-5), suggesting a substantial synthesis of this amino acid in LMB. In addition, the survival rate of LMB fed the 12.5% lipid diet was lower than that of LMB fed the 10% lipid diet (Table V-5). The growth performance of some carnivorous fish is impaired when their diets contain more than 15% starch (NRC 2011). For example, Li et al. (2015) reported that the growth of giant croaker (*Nibea japonica*) decreased with increasing the dietary cornstarch level to more than 12.2%. In addition, juvenile grouper (*Epinephelus akaara*), a dietary level of 7.64% starch appeared to be sufficient for maximum growth (Wang et al. 2016). Similarly, we found that even 10% of dietary starch could limit the weight gain (Table V-2) of LMB. There are reports that the requirement of LMB for dietary lipids is about 10-15%, dependent on experimental conditions (Huang et al. 2017; Bright et al. 2005). Results of our study indicated that LMB grew faster when fed a 10% lipid diet,

compared with a 7.5% lipid diet (Chapter IV). Taken together, we recommend that the requirement of juvenile LMB for dietary lipids be 10%. For comparison, omnivorous fish (e.g., tilapia) tolerate high dietary starch intake well, as increasing the dietary starch level from 10% to 40% improves their growth performance (Amirkolaie et al. 2006), whereas some carnivores (e.g., hybrid grouper) tolerate well 28% cornstarch in diets and do not accumulate excessive intraperitoneal fat, compared with a 0% starch diet (Luo et al. 2016). Likewise, many species of fish have dietary requirements for 15-20% starch for optimal growth (Watanabe 1982). The lower limit to the inclusion of starch and lipids in LMB diets is consistent with our finding that the major tissues of LMB, including the intestine, liver, skeletal muscle, and kidneys, had a low ability to oxidize glucose and fatty acids into CO_2 (Chapter II and III).

The primary reason for the lower rate of growth in LMB fed the 10-15% starch diets compared with the 5% starch diet or in LMB fed the 10% lipid diet compared with the 12.5% lipid diet may result from metabolic and structural alterations in the liver of the LMB. To our knowledge, such a phenomenon has not been reported for LMB. We found that increasing the dietary starch from 5 to 15% or the dietary lipid level from 10 to 12.5% did not affect the relative feed intake (mg feed/g body weight per day) of the LMB. We found that after the second week until the end of the trial, daily feed intake per fish decreased with increasing dietary starch or lipid levels. Like land animals (Wu 2018), fish usually regulate their feed consumption to meet energy requirements (Hemre et al. 1995; Sveier et al. 1999). When fed a diet containing excess energy, fish exhibit growth depression due to a reduction in feed (Ellis and Reigh 1991; El-Sayed and Garling 1988; Han et al. 2014a) and protein intakes (Khan and Abidi 2012). Likewise, high dietary starch or lipid levels, along with high dietary energy levels, decrease protein intake by animals, which contributes to reduced growth rate, as observed for LMB in this study. Moreover, as in land

animals (Wu 2020), reduced feed intake by fish may result from pathological changes in tissues of fish (e.g., the intestine and liver) due to excessive starch and lipid intakes (Tan et al. 2007). A decrease in growth rate in association with excessive accumulation of glycogen in the liver (hepatic glycogenosis) was observed in some fish species (e.g., ≥ 10% starch for juvenile grouper and ≥ 7% glucose for juvenile white sturgeon) fed diets containing high starch levels (Wang et al. 2016; Fynn-Aikins et al. 1992). Clearly, LMB cannot tolerate ≥ 10% dietary starch and > 10% dietary lipids (dry matter basis).

The FCR and PER in LMB were not affected by the levels of dietary starch (5-15%) or lipids (10-12.5%). Our results suggest that these dietary levels of starch and lipids do not negatively influence the efficiency of the utilization of dietary nutrients for weight gains of LMB during a 56-day experimental period. However, when the components of weight gain are unknown, values on the FCR and PER may be misleading. For example, we noted that increasing the dietary starch level from 5 to 15% reduced the retention of dietary protein in LMB by 3.2% and increased the retention of dietary lipids in the body by 27% (Table V-4), possibly contributing to an unfavorably fatter carcass. Similarly, Lie et al. (1988) suggested that protein retention is a better indicator of feed efficiency for lean tissue growth than the PER in fish species. Likewise, Han et al. (2014a) concluded that a body weight change cannot be regarded as an accurate predictor of true protein accretion in fish when their bodies accumulate lipids. It is possible that high dietary starch impairs the digestion of dietary protein and/or the subsequent synthesis of protein from AAs in extra-intestinal tissues, while promoting lipid synthesis from starch-derived glucose in tissues, such as the liver and skeletal muscle (Table V-10) as well as white adipose tissue depots (Table V-7). Nonetheless, a lower rate of growth in fish (including LMB) will delay the time from their entry into aquaculture facilities to their marketing. This will increase both the costs of farm labor

as well as the risks for production losses due to possible infectious diseases, climate changes, and natural disasters, thereby leading to reductions in production efficiency and economic returns. Therefore, excessive levels of starch and lipids in fish diets should always be avoided.

White adipose tissue, which is distributed in abdominal, mesenteric, subcutaneous, and peri-gonadal sites, as well as the liver, skeletal muscle, and other organs, is the main depot for lipid storage primarily as triacylglycerols in fish (Salmerón 2018), as in land animals (Wu 2018). Juvenile LMB contained 6.33% to 7.66% lipids (Table V-8). Based on the content of lipids in skeletal muscle (1.19% to 1.36%; Table V-8) and assuming that skeletal muscle represents about 50% of the body weight, about 9% of total lipids in LMB are present in the skeletal muscle of the fish (1.19% x 0.5/6.33% = 9.4%; 1.36% x 0.5/7.66% = 8.9%). Considering that lipid content in white adipose tissue is 82% (g/g; Jobgen et al. 2009) and that IPFR was 2.24% to 2.78% of body weight in LMB (Table V-7), abdominal white adipose tissue contributed to about 30% (2.24% x 0.82/6.33% = 28.4%; 2.78% x 0.82/7.66% = 29.8%) of total lipids in the fish. Thus, abdominal adipose tissue is a main site of fat deposition in juvenile LMB. Increasing the dietary starch level from 5 to 15% or dietary lipid level from 10 to 12.5% promoted the accumulation of lipids in the abdominal cavity, these results indicate that the nutritional regulation of fat accretion in LMB is depot-specific. At present, it is unknown whether the enlarged fat mass in the body results from an increase in the volume of existing adipocytes (hypertrophy) and/or the number of adipocytes through the proliferation and differentiation of precursor cells (hyperplasia or adipogenesis). Future studies are warranted to address this issue.

In monogastric animals (e.g., swine, poultry, and rats), chronic high intake of dietary starch or lipids results in the development of fatty liver and hepatic dysfunction (Wu 2020). This has been assumed to occur in fish. However, we observed that increasing the dietary starch level from 5 to

15% or the dietary lipid level from 10 to 12.5% did not increase lipid content in the liver of LMB but rather reduced lipid content in this organ (Table V-9) likely due to reduced deposition of dietary lipids. Based on the retention of dietary lipids in LMB (Table V-4) and their digestibility (90%), 38% to 49% of absorbed dietary lipids are oxidized to CO_2 or excreted from the body. Notably, high dietary starch resulted in hepatic glycogenosis (Figures V-1 and 2). Thus, the hepatopathy in LMB fed high starch diets is not a fatty liver disease. In support of this view, we observed that: (1) the HSI, hepatocyte area, and hepatic glycogen concentrations increased substantially with increasing the dietary starch level from 5 to 15% (Tables V- 7 and 9; Figure V-1); and (2) excessive amounts of glycogen deposits are present in the cytosol of hepatocytes, as determined by the PAS stain (Figure V-1). The enlargement of the liver in LMB fed the 10-15% starch diets is due, in part, to the deposition of water because 1 g glycogen is associated with 3 g water (Brooks and Fahey 1984), leading to structural abnormalities in the liver and possibly impairments in some of its metabolic functions (e.g., protein synthesis, bile production, and lipoprotein assembly). Our other study revealed that LMB fed diets with low protein and high starch exhibited hepatic glycogenosis. Similar results were obtained in the present study where LMB were fed diets containing 45% protein. Thus, high intake of starch can induce hepatic glycogenosis in LMB regardless of low or adequate protein intake. This species is highly sensitive to starch intake because it has a limited ability to oxidize glucose in the body (Chapter II). Of note, Goodwin et al. (2002) reported that juvenile LMB fed a 45% protein diet containing 21%, 27% or 35% carbohydrate (nitrogen-free extract that included starch). In contrast, Amoah et al. (2008) did not detect any differences in the PER, hepatosomatic index, or liver glycogen concentration among fish fed the diets containing 13, 19, and 25% starch. This may be explained, based on the findings from the present study, that the control, 13% starch diet might have maximized its adverse effects on these variables.

The mechanisms responsible for the hepatic glycogenosis in LMB fed a diet containing ≥ 10% starch are not fully understood. There is a suggestion that some fish develop this disease due to their limited ability to control blood glucose concentration (Palmer et al. 1972; NRC 2011). In many fish species (Kamalam et al. 2017), as in land animals (Wu 2020), glucose can be converted into glycogen through glycogenesis in the liver and skeletal muscle. This was also observed in LMB (Table V-10). Of particular note, the rates of glycogen synthesis from glucose in the liver of LMB were much greater than those for fatty acid synthesis (Table V-10), indicating a high capacity for hepatic glycogenesis in LMB. In this study, we also found that high dietary starch resulted in 84% to 130% increases in serum glucose concentrations at 2 h after feeding in comparison with time 0 (24 h after feeding) and the elevated glucose levels remained through 8 h after feeding (Table V-11). Such a prolonged post-prandial elevation in the circulating levels of glucose is not common in healthy terrestrial animals (Wu 2018) and is a characteristic of metabolism in LMB, possibly due to a low uptake of glucose by extra-hepatic tissues, including skeletal muscle and kidneys (Li and Wu 2019). Carnivorous fish generally exhibit prolonged hyperglycemia following carbohydrate-rich meals, with glucose a weak insulin secretagogue (Kamalam et al. 2017). At the same extracellular glucose concentration (5 mM), the rate of conversion of glucose into glycogen in the liver did not differ among LMB fed the 5%, 10% and 15% starch diets, indicating that the machinery of hepatic glycogenesis (i.e., enzymes and regulation) was not altered by high starch intake. Thus, elevated blood glucose, which is derived from intestinal absorption after feeding, increased the intake of glucose by the liver to drive hepatic glycogen synthesis because of enhanced substrate provision. This leads to hepatic glycogenosis. Such a metabolic disease not only reduces the feed intake of LMB and impairs their growth, but also causes their death (Goodwin et al. 2002).

In conclusion, the retention of dietary protein in LMB was affected by dietary starch or lipid levels and their interactions. The liver of LMB had a high capacity for synthesizing glycogen from glucose and a much lower capacity for converting glucose into fatty acids. Compared with the liver, the synthesis of fatty acids or glycogen from glucose was limited in the skeletal muscle of LMB. A dietary level of starch ≥10% resulted in hepatic glycogenosis due to prolonged elevations of blood glucose for glycogen synthesis in the liver, as well as reductions in the feed intake and weight gain of LMB. Compared with a 10% lipid diet, a dietary level of 12.5% lipid decreased the feed intake, growth and survival of the fish. Increasing the dietary starch level from 5 to 15% or the lipid level from 10 to 12.5% did not lead to a fatty liver. Based on these results, we recommend dietary lipid and starch levels to be 10% and < 10% (dry matter basis), respectively, for juvenile LMB to maximize the retention of dietary protein in their bodies.

Table V-1 Composition of the experimental diets

Ingredient (%, DM basis)	L10/C5	L10/C10	L10/C20	L12.5/C5	L12.5/C10	L12.5/C20
Fish meal Menhaden[1]	53.73	53.73	53.73	53.73	53.73	53.73
SPC[2]	12.62	12.62	12.62	12.62	12.62	12.62
Soybean oil: fish oil (2:1)[3,5]	3.00	3.00	3.00	3.00	3.00	3.00
Poultry fat[4]	1.23	1.23	1.23	3.73	3.73	3.73
Dextrinized starch[6]	5.00	10.00	15.00	5.00	10.00	15.00
Vitamin premix[7]	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[8]	2.00	2.00	2.00	2.00	2.00	2.00
Cellulose[9]	19.18	14.18	9.18	16.68	11.68	6.68
CMC[10]	2.00	2.00	2.00	2.00	2.00	2.00
Choline chloride	0.24	0.24	0.24	0.24	0.24	0.24
Composition (%, DM basis) *						
Crude protein	45.0	45.0	45.0	45.0	45.0	45.0
Lipids	10.0	10.0	10.0	12.5	12.5	12.5
Energy (kJ/g)	14.96	15.82	16.68	15.94	16.80	17.66

* The content of dry matter (DM) in the diets (as-fed diets) was 97.0%.

[1] Omega Fish Meal (Corporate Headquarters of Omega Protein, Houston, Texas); [2] PROFINE® Soy Protein Concentrate (Dupont, WI, USA); [3] Nutrioli Pure Soybean Oil (Ragasa, N.L., Mexico); [4] Chicken fat (Tyson Foods, Arkansas, USA); [5] Fish oil (Paragon, Illinois, USA).

[6] Maltodextrin (Baolingbao Biology, Shangdong, China).

[7] Vitamin premix (g/kg): vitamin A, 2.31; vitamin D_3, 2.02; vitamin E, 20.00; vitamin K_3, 1.2; vitamin C, 30.00; vitamin B_5, 10.87; inositol, 15.00; niacin, 14.00; vitamin B_6, 3.04; vitamin B_2, 3.00; vitamin B_1, 3.26; biotin, 0.15; folic acid, 0.6; vitamin B_{12}, 0.02; Cellulose, 894.53.

8 Mineral premix (g/kg): NaCl, 181.94; $MgSO_4.7H_2O$, 293.33; $FeSO_4.7H_2O$, 11.11; $AlCl_3.6H_2O$, 0.33; KI, 0.0.33; $CuSO_4.5H_2O$, 1.11; $MnSO_4$, 2.33； $CoCl.6H_2O$, 0.43; $ZnSO_4.7H_2O$, 9.04; Na_2SeO_3, 0.33; Cellulose, 500.00.

[9] Microcrystaline cellulose 102 (Blue Diamond Growers, California, USA).

[10] Sodium carboxy methyl cellulose (Pro Supply Outlet, California, USA).

Table V-2 Growth performance of juvenile LMB fed diets containing different starch and lipid levels

Diet		Body weight (BW, g/fish)					Weight gain over the initial BW (%)			
Starch (S, %)	Lipids (L, %)	Day 0	Day 14	Day 28	Day 42	Day 56	Days 0 - 14	Days 0 - 28	Days 0 - 42	Days 0 - 56
5	10	4.86	9.10	14.4^{a}	20.6^{a}	26.7^{a}	87.2	197^{a}	323^{a}	448^{a}
10	10	4.87	9.24	14.8^{a}	20.6^{a}	26.4^{a}	90.0	203^{a}	323^{a}	443^{a}
15	10	4.87	9.06	13.8^{b}	20.1^{a}	24.9^{b}	86.0	183^{b}	313^{a}	412^{bc}
5	12.5	4.85	9.16	13.8^{b}	20.4^{a}	25.5^{ab}	88.8	185^{b}	320^{a}	425^{ab}
10	12.5	4.80	9.09	13.5^{b}	18.2^{b}	23.1^{c}	89.3	182^{b}	278^{b}	381^{d}
15	12.5	4.85	8.97	13.9^{b}	19.0^{b}	24.1^{bc}	84.8	186^{b}	291^{b}	391^{cd}
Pooled SEM		0.02	0.04	0.13	0.25	0.39	0.80	2.80	4.97	8.09
Main effect means										
5		4.86	9.13	14.1	20.5^{A}	26.1^{A}	88.0	191	321^{A}	437^{A}
10		4.84	9.17	14.1	19.4^{B}	24.8^{B}	89.7	193	301^{B}	412^{B}
15		4.86	9.00	13.8	19.6^{B}	24.4^{B}	85.4	184	302^{B}	401^{B}
	10	4.87	9.14	14.3^{A}	20.4^{A}	26.0^{A}	87.7	194^{A}	320^{A}	434^{A}
	12.5	4.84	9.06	13.7^{B}	19.2^{B}	24.2^{B}	87.6	184^{B}	297^{B}	399^{B}
Two-way ANOVA										
S		0.271	0.219	0.203	0.001	0.007	0.105	0.278	0.003	0.018
L		0.573	0.152	0.005	0.029	0.005	1.000	0.023	0.038	0.010
S × L		0.089	0.066	0.404	0.428	0.208	0.0084	0.246	0.373	0.156

[1] Values are means with pooled SEM for 3 tanks of fish per dietary group.
[a-c]: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).
[A-C]: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table V-3 Feed intake, feed conversion ratio, and protein efficiency ratio (PER) of juvenile LMB fed diets containing different starch and lipid levels[1]

Diet		Feed intake (FI, g/fish)				Feed conversion ratio (FI/weight gain)				PER (weight gain/feed intake, g/g)			
Starch (S, %)	Lipids (L, %)	Days 0-14	Days 14-28	Days 28-42	Days 42-56	Days 0-14	Days 14-28	Days 28-42	Days 42-56	Days 0-14	Days 14-28	Days 28-42	Days 42-56
5	10	3.88	6.07^{a}	6.43^{a}	7.93^{a}	0.92	1.15	1.04	1.30	2.43^{a}	1.94	2.14	1.71
10	10	3.96	5.89^{a}	5.90^{a}	7.10^{ab}	0.91	1.06	1.02	1.22	2.45^{a}	2.10	2.18	1.81
15	10	4.10	5.37^{b}	5.84^{a}	6.68^{ab}	0.98	1.13	0.95	1.39	2.27^{b}	1.96	2.40	1.60
5	12.5	3.90	5.51^{b}	6.28^{a}	6.95^{ab}	0.91	1.19	0.95	1.36	2.45^{a}	1.87	2.33	1.63
10	12.5	3.92	4.99^{b}	4.61^{b}	6.38^{b}	0.92	1.13	0.98	1.30	2.43^{a}	1.96	2.27	1.71
15	12.5	3.78	5.17^{b}	4.73^{b}	6.21^{b}	0.92	1.05	0.95	1.20	2.42^{a}	2.12	2.40	1.85
Pooled SEM		0.05	0.10	0.16	0.21	0.02	0.03	0.02	0.03	0.03	0.05	0.05	0.05
Main effect means													
5		3.89	5.79^{A}	6.36^{A}	7.44^{A}	0.91	1.17	0.99	1.18	2.45	1.91	2.24	1.67
10		3.94	5.44^{B}	5.26^{B}	6.74^{B}	0.91	1.10	1.00	1.18	2.44	2.03	2.22	1.76
15		3.94	5.27^{B}	5.28^{B}	6.40^{B}	0.95	1.09	0.95	1.17	2.35	2.04	2.40	1.72
	10	3.98	5.78^{A}	6.06^{A}	7.24^{A}	0.93	1.11	1.00	1.19	2.38	2.00	2.24	1.71
	12.5	3.87	5.22^{B}	5.21^{B}	6.48^{B}	0.91	1.12	0.96	1.17	2.44	1.98	2.33	1.73
Two-way ANOVA													
S		0.269	0.001	0.002	0.024	0.735	0.979	0.983	0.999	0.971	0.929	0.962	0.992
L		0.839	0.003	0.001	0.009	0.945	0.982	0.217	0.644	0.204	0.781	0.221	0.781
S × L		0.289	0.171	0.120	0.815	0.414	0.075	0.452	0.790	0.437	0.124	0.458	0.635

[1] Values are means with pooled SEM for 3 tanks of fish per dietary group. a-c: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

[A-C]: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table V-4 Retention (or productive value, %) of dietary energy and nutrients in the body of juvenile largemouth bass fed diets containing different starch and lipid levels.

Diet		Retention or productive value of dietary nutrients and energy, %				
Starch (S) %	Lipids (L) %	Energy	Protein	Lipids	Phosphorus	Calcium
5	10	39.14^{b}	29.74^{c}	52.62^{d}	35.00^{c}	49.02^{ab}
10	10	41.50^{a}	33.17^{a}	66.23^{b}	39.89^{a}	50.66^{a}
15	10	39.88^{b}	29.88^{c}	71.36^{a}	35.76^{c}	46.87^{c}
5	12.5	39.52^{b}	31.86^{b}	49.69^{de}	37.80^{b}	48.68^{b}
10	12.5	35.38^{d}	29.19^{c}	48.44^{e}	35.00^{c}	46.16^{c}
15	12.5	36.59^{c}	29.74^{c}	58.24^{c}	37.30^{b}	49.56^{ab}
Pooled SEM		0.23	0.20	1.01	0.24	0.46
Main effect means						
5		39.33	30.80^{A}	51.13^{C}	36.66	49.02
10		38.43	31.19^{A}	57.33^{B}	37.44	48.40
15		38.24	29.82^{B}	64.80^{A}	36.53	48.21
	10	30.17^{A}	30.93^{A}	63.38^{A}	37.04	48.96
	12.5	36.82^{B}	30.27^{B}	52.12^{B}	36.70	48.12
Two-way ANOVA						
S		0.177	0.010	< 0.001	0.293	0.209
L		< 0.001	0.032	< 0.001	0.494	0.561
S × L		< 0.001	< 0.001	0.032	< 0.001	0.020

[1] Values are means with pooled SEM for 3 tanks per dietary group.
[a-c]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
[A-C]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

Table V-5 Percentages (%) of the gains of nutritionally dispensable amino acids to their dietary intakes in juvenile largemouth bass fed diets containing different starch and lipid levels

Lipids (L) %	Starch (S) %	Ala	Asn	Asp	Cys	Gln	Glu	Gly	Pro	OH-Pro	Ser	Tyr
10	5	29.11^{d}	28.42^{e}	23.70^{e}	34.22^{d}	23.96^{e}	26.54^{e}	40.49^{e}	33.37^{e}	31.57^{e}	28.92^{e}	24.04^{d}
10	10	32.82^{a}	32.04^{a}	26.71^{a}	38.58^{a}	27.02^{a}	29.93^{a}	45.65^{a}	36.95^{a}	35.60^{a}	32.60^{a}	27.11^{a}
10	15	30.06^{cd}	29.34^{de}	24.46^{de}	35.33^{cd}	24.74^{de}	27.40^{de}	41.81^{de}	34.45^{d}	32.60^{de}	29.86^{de}	24.82^{cd}
12.5	5	32.10^{ab}	31.33^{ab}	26.13^{ab}	37.31^{b}	26.43^{ab}	29.27^{ab}	44.65^{ab}	36.79^{ab}	34.82^{ab}	31.89^{ab}	26.51^{ab}
12.5	10	30.54^{c}	29.81^{cd}	25.20^{cd}	36.42^{bc}	25.14^{cd}	28.23^{cd}	43.09^{cd}	35.50^{cd}	33.12^{cd}	30.34^{cd}	25.22^{c}
12.5	15	31.18^{bc}	30.43^{bc}	25.38^{bc}	36.65^{b}	25.67^{bc}	28.43^{bc}	43.37^{bc}	35.74^{bc}	33.82^{bc}	30.97^{bc}	25.75^{bc}
Pooled SEM		0.35	0.34	0.27	0.37	0.28	0.30	0.46	0.35	0.37	0.34	0.32
Main effect means												
	5	30.61	29.88	24.58^{B}	35.77^{B}	25.19	27.91^{B}	42.57^{B}	34.41^{B}	33.19	30.40	25.28
	10	31.68	30.92	25.96^{A}	37.50^{A}	26.08	29.08^{A}	44.37^{A}	36.23^{A}	34.36	31.47	26.17
	15	30.62	29.89	24.92^{B}	35.99^{B}	25.20	27.92^{B}	42.59^{B}	35.09^{AB}	33.21	30.41	25.29
10		30.66	29.93	24.96	36.05	25.24	27.96	42.65	34.92	33.26	30.46	25.33
12.5		31.27	30.53	25.35	36.79	25.74	28.64	43.70	35.56	33.92	31.06	25.83
Two-way ANOVA												
S		0.088	0.092	0.015	0.003	0.077	0.017	0.013	0.007	0.084	0.085	0.089
L		0.137	0.169	0.155	0.058	0.143	0.055	0.050	0.163	0.136	0.151	0.145
S × L		0.001	0.001	0.001	<0.001	0.001	<0.001	<0.001	0.015	0.001	0.001	0.001

[1] Values are means with pooled SEM for 3 tanks per dietary group.
[a-c]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
[A-C]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

Table V-6 Retention (%) of ingested nutritionally indispensable amino acids in juvenile largemouth bass fed diets containing different starch and lipid levels

Lipids (L) %	Starch (S) %	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val
10	5	25.84^{c}	28.16^{d}	25.41^{b}	25.87^{c}	23.04^{d}	19.12^{d}	26.26^{d}	27.03^{d}	26.87^{d}	25.58^{d}
10	10	28.45^{a}	31.75^{a}	27.43^{a}	27.96^{ab}	25.98^{a}	21.56^{a}	29.61^{a}	30.47^{a}	30.30^{a}	28.84^{a}
10	15	26.67^{bc}	29.08^{cd}	25.53^{b}	26.71^{bc}	23.79^{cd}	19.75^{cd}	27.11^{cd}	27.90^{cd}	27.75^{cd}	26.41^{cd}
12.5	5	28.49^{a}	31.06^{ab}	27.26^{a}	28.53^{a}	25.41^{ab}	21.09^{ab}	28.96^{ab}	29.80^{ab}	29.64^{ab}	28.20^{ab}
12.5	10	27.10^{bc}	29.54^{bd}	25.94^{b}	27.14^{bc}	24.17^{bd}	20.06^{bcd}	27.55^{bd}	28.35^{bcd}	28.19^{bcd}	27.21^{bc}
12.5	15	27.67^{ab}	30.16^{bc}	26.48^{ab}	27.71^{ab}	24.68^{bc}	20.48^{ac}	28.13^{bc}	28.95^{ac}	28.78^{ac}	27.39^{bc}
Pooled SEM		0.28	0.33	0.25	0.27	0.27	0.23	0.31	0.32	0.32	0.29
Main effect means											
	5	27.16	29.61	26.34	26.70	24.23	20.11	27.61	28.41	28.26	26.89^{B}
	10	27.78	30.65	26.68	27.55	25.08	20.81	28.58	29.41	29.25	28.02^{A}
	15	27.17	29.62	26.00	27.21	24.24	20.11	27.62	28.42	28.27	26.90^{B}
10		26.99	29.66	26.12	26.85	24.27	20.14	27.66	28.47	28.31	26.94
12.5		27.76	30.25	26.56	27.46	24.76	20.54	28.21	29.03	28.87	27.60
Two-way ANOVA											
S		0.350	0.079	0.325	0.709	0.086	0.070	0.076	0.087	0.079	0.022
L		0.084	0.141	0.245	0.155	0.159	0.147	0.167	0.156	0.151	0.127
S × L		0.004	0.001	0.007	0.080	0.001	<0.001	0.001	0.001	0.001	<0.001

[1] Values are means with pooled SEM for 3 tanks per dietary group.
[a-c]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
[A-C]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

Table V-7 The viscerosomatic index (VSI), hepatosomatic index (HSI), intraperitoneal fat ratio (IPFR), hepatocyte size, and survival of juvenile LMB fed diets containing different starch and lipid levels[1].

Diet						
Starch (S, %)	Lipids (L, %)	VSI %	HSI %	Hepatocyte size (μm^2)	IPFR %	Survival %
5	10	7.31^{e}	1.12^{b}	64.2^{e}	2.19^{c}	100
10	10	7.62^{d}	1.76^{a}	75.6^{d}	2.19^{c}	96.3
15	10	8.05^{b}	1.76^{a}	104^{bc}	2.62^{b}	100
5	12.5	7.31^{e}	1.23^{b}	96.3^{c}	2.29^{c}	94.4
10	12.5	7.73^{c}	1.65^{a}	133^{a}	2.91^{a}	92.6
15	12.5	8.54^{a}	1.90^{a}	114^{b}	2.95^{a}	92.6
Pooled SEM		0.10	0.03	2.30	0.05	1.4
Main effect means						
5		7.31^{C}	1.18^{C}	69.9^{C}	2.24^{C}	97.2
10		7.67^{B}	1.71^{B}	100^{B}	2.56^{B}	94.5
15		8.29^{A}	1.83^{A}	123^{A}	2.78^{A}	96.3
	10	7.66	1.55	100	2.34^{B}	98.8^{A}
	12.5	7.86	1.60	95	2.72^{A}	93.2^{B}
Two-way ANOVA						
S		< 0.001	< 0.001	< 0.001	< 0.001	0.161
L		0.349	0.514	0.121	< 0.001	0.0011
S × L		0.605	0.302	0.001	0.066	0.072

[1] Values are means with pooled SEM for 12 fish per dietary group for VSI, HSI, and IPFR. Values are means with pooled SEM for 6 fish per dietary group for hepatocyte size.

[a-c]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

[A-C]: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table V-8 Composition of the whole body and skeletal muscle of juvenile LMB fed diets containing different starch and lipid levels

Diet		Nutrients in the whole body (% of wet weight)					Nutrients in skeletal muscle (% of wet weight)		
Starch (S, %)	Lipids (L, %)	Water	Protein	Lipids	Phosphorus	Calcium	Water	Protein	Lipids
Initial fish									
5	10	72.5^{a}	16.2^{c}	5.83^{c}	0.70^{c}	1.12^{d}	77.1	20.0	1.12^{b}
10	10	70.5^{c}	17.7^{bc}	6.53^{bc}	0.77^{a}	1.25^{a}	77.2	19.9	1.17^{b}
15	10	71.9^{b}	16.7^{b}	6.81^{b}	0.73^{b}	1.19^{bc}	77.0	20.0	1.37^{a}
5	12.5	70.9^{c}	17.7^{a}	6.82^{b}	0.75^{a}	1.22^{ab}	77.1	19.8	1.25^{ab}
10	12.5	70.2^{c}	16.9^{b}	8.22^{a}	0.72^{b}	1.17^{c}	77.4	19.7	1.16^{b}
15	12.5	70.5^{c}	17.0^{b}	7.94^{a}	0.76^{a}	1.23^{ab}	77.3	19.8	1.34^{a}
Pooled SEM		0.17	0.15	0.25	0.006	0.014	0.049	0.051	0.040
Main effect means									
5		71.7^{A}	16.8	6.33^{B}	0.72	1.17	77.1	19.9	1.19^{B}
10		70.4^{B}	17.3	7.37^{A}	0.75	1.21	77.3	19.8	1.16^{B}
15		71.2^{A}	16.9	7.63^{A}	0.75	1.21	77.2	19.9	1.36^{A}
	10	71.6	16.8	6.58^{B}	0.73	1.19	77.1	20.0^{A}	1.22
	12.5	70.5	17.1	7.66^{A}	0.75	1.20	77.3	19.7^{B}	1.25
Two-way ANOVA									
S		< 0.001	0.079	0.005	0.187	0.451	0.199	0.569	0.027
L		0.005	0.389	0.002	0.205	0.550	0.111	0.017	0.705
S × L		0.295	0.044	0.676	0.011	0.042	0.282	0.988	0.670

[1] Values are means with pooled SEM, n = 12 fish per dietary group.
[a-c]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).
[A-C]: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table V-9 Composition of the liver of juvenile LMB fed with diets containing different protein levels.

Diet		Nutrients in the liver (g/100 g of wet weight)			
Starch (S, %)	Lipids (L, %)	Water	Protein	Lipids	Glycogen
5	10	76.0^{b}	12.9^{a}	2.76^{a}	8.16^{e}
10	10	74.1^{c}	11.2^{b}	2.25^{c}	10.8^{b}
15	10	74.5^{c}	10.1^{c}	1.66^{e}	12.3^{a}
5	12.5	76.9^{a}	11.2^{b}	2.55^{b}	9.12^{d}
10	12.5	75.9^{b}	10.5^{c}	1.87^{d}	9.91^{c}
15	12.5	73.0^{d}	8.90^{d}	1.59^{e}	12.1^{a}
Pooled SEM		0.21	0.15	0.06	0.25
Main effect means					
5		73.7^{C}	12.0^{A}	2.66^{A}	8.65^{C}
10		75.0^{B}	10.9^{B}	2.06^{B}	10.4^{B}
15		76.4^{A}	9.5^{C}	1.62^{C}	12.2^{A}
	10	74.8	11.4^{A}	2.22^{A}	10.4
	12.5	75.3	10.2^{B}	2.00^{B}	10.4
Two-way ANOVA					
S		0.001	< 0.001	< 0.001	< 0.001
L		0.119	0.002	0.020	1.000
S × L		0.014	0.414	0.589	0.366

[1] Values are means with pooled SEM, n = 12 fish per dietary group.

[a-c]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

[A-C]: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table V-10 Syntheses of fatty acids and glycogen from glucose in the liver and skeletal muscle of juvenile LMB fed diets containing different starch and lipid levels

Diet		*Liver (nmol C/2h/mg protein)*		*Skeletal muscle (nmol C/2h/mg protein)*	
Starch (S, %)	Lipids (L, %)	Fatty acids	Glycogen	Fatty acids	Glycogen
5	10	3.26	10.7^{a}	0.68^{c}	1.33
10	10	2.75	10.3^{ab}	0.75^{c}	1.33
15	10	2.63	9.50^{b}	0.96^{b}	1.27
5	12.5	3.19	9.14^{bc}	0.86^{b}	1.23
10	12.5	2.77	8.40^{c}	0.93^{b}	1.37
15	12.5	3.29	9.28^{bc}	1.07^{a}	1.43
Pooled SEM		0.12	0.31	0.03	0.04
Main effect means					
5		3.23	9.92	0.77^{C}	1.28
10		2.76	9.35	0.85^{B}	1.35
15		3.11	9.39	1.01^{A}	1.35
	10	2.88	10.2^{A}	0.80^{B}	1.31
	12.5	3.08	8.94^{B}	0.95^{A}	1.34
Two-way ANOVA					
S		0.285	0.325	0.007	0.686
L		0.398	0.012	0.014	0.655
S × L		0.398	0.505	0.847	0.411

[1] Values are means with pooled SEM, n = 9 fish per dietary group.

[a-c]: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

[A-C]: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

Table V-11 Concentrations of lactate and glucose in the serum of juvenile LMB fed diets containing different starch and lipid levels.

Diet		Lactate (mM)					Glucose (mM)				
Starch (S, %)	Lipids (L, %)	2h	4h	8h	12h	24h	2h	4h	8h	12h	24h
5	10	2.58^{b}	1.55^{c}	1.70^{bc}	1.47^{b}	1.78^{c}	4.70^{b}	3.76^{b}	2.99^{bc}	1.49^{b}	1.63^{d}
10	10	2.59^{b}	1.49^{c}	2.01^{b}	1.51^{b}	1.96^{c}	4.78^{b}	3.37^{b}	3.67^{b}	2.06^{a}	1.90^{cd}
15	10	3.26^{a}	2.86^{a}	2.47^{a}	3.06^{a}	3.17^{a}	5.45^{a}	5.57^{a}	4.57^{a}	2.19^{a}	2.17^{bc}
5	12.5	2.49^{b}	1.83^{c}	1.39^{c}	1.65^{b}	1.91^{c}	3.92^{c}	3.38^{b}	2.62^{c}	1.49^{b}	2.14^{bc}
10	12.5	2.78^{b}	2.39^{b}	2.69^{a}	2.59^{a}	2.66^{b}	3.96^{c}	3.56^{b}	3.08^{bc}	1.79^{ab}	2.29^{b}
15	12.5	2.69^{b}	2.32^{b}	2.57^{a}	2.79^{a}	3.28^{a}	5.01^{ab}	4.02^{b}	3.55^{b}	1.86^{ab}	2.58^{a}
Pooled SEM		0.09	0.12	0.12	0.15	0.14	0.17	0.18	0.17	0.13	0.09
Main effect means											
5		2.54^{B}	1.69^{B}	1.54^{B}	1.56^{C}	1.85^{C}	4.31^{B}	3.46^{B}	2.81^{C}	1.49^{B}	1.88^{B}
10		2.69^{B}	1.94^{B}	2.35^{A}	2.05^{B}	2.31^{B}	4.37^{B}	3.57^{B}	3.37^{B}	1.93^{A}	2.09^{B}
15		2.98^{A}	2.59^{A}	2.52^{A}	2.93^{A}	3.22^{A}	5.23^{A}	4.80^{A}	4.06^{A}	2.03^{A}	2.37^{A}
	10	2.81	1.97	2.06	2.02	2.31	4.98^{A}	4.23^{A}	3.74^{A}	1.92	1.90^{B}
	12.5	2.65	2.10	2.22	2.34	2.62	4.30^{B}	3.53^{B}	3.08^{B}	1.72	2.34^{A}
Two-way ANOVA											
S		0.011	0.001	<0.001	<0.001	<0.001	0.004	0.002	0.001	0.023	0.030
L		0.325	0.329	0.254	0.151	0.154	0.017	0.009	0.010	0.454	0.008
S × L		0.157	<0.001	0.028	0.004	0.200	0.869	0.006	0.519	0.863	0.934

[1] Values are means with pooled SEM, n = 18 fish per dietary group at each time point. a-c: Within a column, means not sharing the same superscript letter differ ($P < 0.05$).

[A-C]: Within a column, main effect means not sharing the same superscript letter differ ($P < 0.05$).

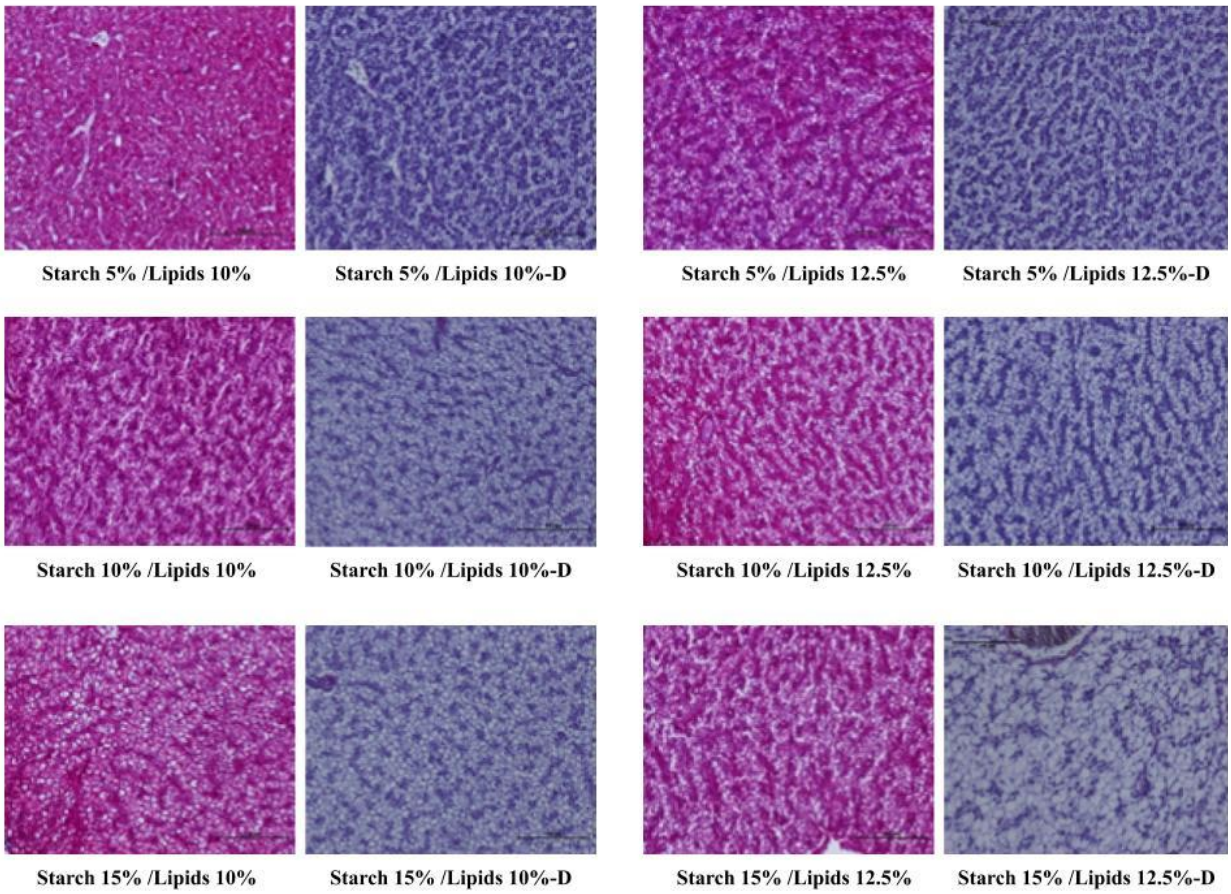


Figure V-1 Hepatic histology of juvenile LMB fed diets containing different starch and lipid levels. The PAS stain (without diastase): showing the red staining of the hepatocyte cytoplasm. D refers to the PAS stain with diastase (to break down glycogen): showing a marked decrease in the bright red staining (or the amount of glycogen) of the hepatocyte cytoplasm.

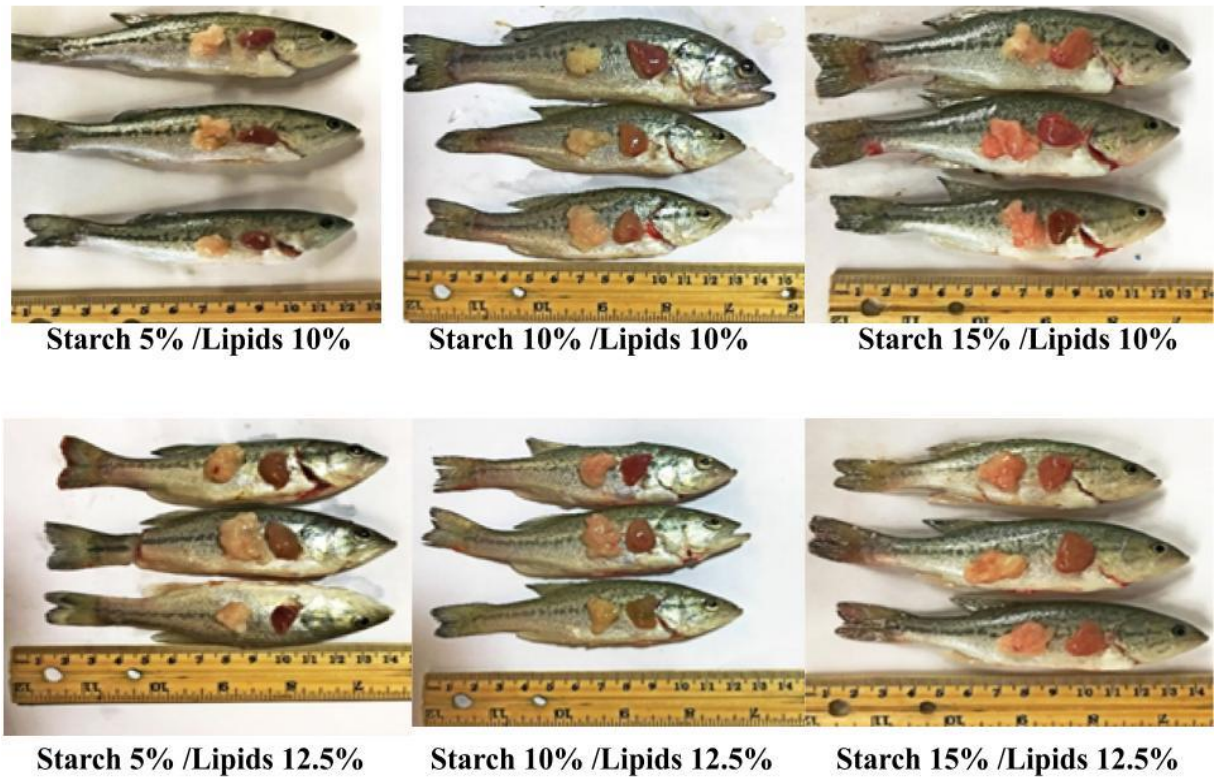


Figure V-2 Representative pictures of the whole fish body, liver, and intraperitoneal adipose tissue in LMB fed diets containing different starch and lipid levels

CHAPTER VI

EFFECTS OF REPLACING FISHMEAL WITH POULTRY BY-PRODUCT MEAL AND SOYBEAN MEAL ON THE GROWTH, FEED UTILIZATION AND HEALTH OF LMB (*MICROPTERUS SALMOIDES*)

Abstract Five isonitrogenous and isocaloric diets [containing 54%, 30%, 15%, 10%, and 5% fishmeal protein, dry matter (DM) basis] were prepared by replacing fishmeal with poultry by-product meal plus soybean meal to feed juvenile largemouth bass (LMB, with an initial mean body weight of 4.9 g) for 8 weeks. All diets contained 54% crude protein (CP) and 13% lipids. There were 4 tanks of fish per treatment group (15 fish/tank). The fish were fed twice daily with the same feed intake (g/fish) in all the dietary groups. Results indicated that the inclusion of 15% fishmeal in the diet is sufficient for LMB growth. However, some of the fish that were fed diets containing ≤ 15% fishmeal protein had black skin syndrome (characterized by the darkening of the skin), as well as damages in the eye, intestine and liver. The concentrations of taurine, methionine, threonine and histidine in serum were reduced ($P < 0.05$) in fish fed the diets containing 5%, 10% and 15% fishmeal protein, compared with the 30% and 54% fishmeal protein diets. Interestingly, the concentrations of tyrosine and tryptophan in serum were higher in fish fed diets with ≤ 15% fishmeal protein than thosein the 54% fishmeal-protein group. These results indicated that 15% fishmeal protein in the diet containing poultry by-product meal and soybean meal was sufficient for the maximum growth and feed efficiency in LMB but inadequate for their intestinal, skin, eye, and liver health. We recommend that the diets of juvenile LMB contain 30% fishmeal protein (dry matter basis).

Introduction

Previous studies indicated that LMB has a high requirement for dietary protein (mainly from fishmeal) (Huang et al. 2017; Li et al. 2019) because the fish prefers to utilize AAs, rather than glucose and fatty acids, as major metabolic fuels (Chapter II and III). Moreover, a protein-sparing effect of lipids and starch was not observed in LMB when they were fed diets containing 40% crude protein (CP) and 12.5% lipids or 22.3% starch, compared with diets containing 45% (or 50% CP), 10% lipids and 9.2% starch (Chapter III and IV). This is possibly due to some metabolic characteristics of the fish, such as the limited oxidation of glucose in skeletal muscle but excess deposition of glycogen in the liver (Chapter II-IV). Despite our inadequate knowledge of protein metabolism in fish, there is an urgent need to reduce the inclusion level of fishmeal in aquafeeds. Aquaculture is faced with such a challenge because the worldwide fishmeal resources are diminishing and its costs are increasing (FAO 2012; Goytortua-Bores et al. 2006; Kaushik and Seiliez 2010) but global fish farming is rapidly expanding to provide high-quality protein for the growing human population (Naylor et al. 2009; Hardy 2010).

Soybean meal is considered to be an economical and nutritious plant feedstuff because of its reasonable price, steady supply, and low phosphorus content relative to fishmeal (Zhou et al. 2005; Biswas et al. 2007; Yang et al. 2011). However, the use of high-plant protein products is beset with some nutritional problems for carnivorous fish like LMB, such as low protein digestibility (Glencross et al. 2004), poor utilization of dietary nutrients including minerals (Bonaldo et al. 2008; Vielma et al. 2000), and an imbalance of AAs (Cheng et al. 2003; Chou et al. 2004). In addition, terrestrial animal by-product meals (e.g., meat and bone meal, blood meal and poultry by-product meals), which contain sufficient amounts and balanced AAs (Li et al. 2011) and confer high palatability (Sabbagh et al. 2019), are considered as feed ingredients of good nutritional

quality (Olsen and Hasan 2012). Through the inclusion of both poultry by-product meal and soybean meal, the level of fishmeal in the diets of LMB could be reduced to 16% without any negative effects on growth or feed utilization (Ren et al. 2018). However, the impact of such diets on the health of fish was not evaluated in that study.

Considering that LMB cannot tolerate high dietary starch levels, high-protein feeds have been used to feed LBM in practical production settings. The purpose of this study was to evaluate the effects of the replacement of fishmeal with soybean meal and poultry by-product meal in diets on the growth, feed utilization, and health of juvenile LMB.

Materials and Methods

Experimental Diets

Five diets were formulated to contain 54% crude protein (CP), 13% lipids, and 18.2 kJ energy/g [(dry matter (DM) basis, Table VI-1]. The gross energy content of the diets was calculated on the basis of 22.6 kJ/g, 39.3 kJ/g and 17.2 kJ/g for protein, lipids, and glycogen/starch, respectively (Wu 2018). Fishmeal-protein content in the diet was gradually reduced to 30%, 15%, 10%, and 5% with the inclusion of soybean meal and poultry by-product meal. The diets were designated as FM30, FM15, FM10, and FM5, respectively. The control diet containing fishmeal as the sole source of protein (54% CP, DM basis) was designated as FM54. All feed ingredients but oils were first thoroughly mixed, followed by the addition of fish oil, soybean oil, and water, to form a moist dough. All experimental diets in the size of 1.5 to 2.0-mm in width and 4 to 6-mm in length were produced by a screw extruder (Big Bite Meat Grinder, West Chester, OH) and dried in an oven at 50 ºC until DM content was 96.0% (Wu et al. 1999). All feeds were stored at -20 ℃ until use within 10 weeks.

Experimental animals

Juvenile LMB (about 2 g) were obtained from a commercial fish farm (Larry's Fish Farm, Giddings, TX, USA) and housed in the Kleberg Center of Texas A&M University, as previously described (Jia et al. 2017). The photoperiod of the housing facility was maintained for 14 h per day, with lights being turned off between 10:00 PM and 8:00 AM. The growth trial was carried out in the Kleberg Center of Texas A&M University (College station, TX, USA). The fish were acclimated to our laboratory conditions and fed a commercial feed (AquaMax® Grower 400, Purina, MO) for 20 days. At the beginning of this trial, fish with a uniform body size (initial body weight of ~4.9 g per fish) were randomly distributed into each tank (15 fish/tank). There were 4 replicate tanks (55 L of water/tank) per dietary group. The feeding experiment lasted for 8 weeks. The photoperiod of the housing facility was maintained at 10 h per day (10:00 PM to 8:00 AM). The quality parameters of water [pH 6.5-7.5, NH_4^+ (< 1 mg/L), nitrite (< 1 mg/L), nitrate (< 20 ppm), and dissolved O_2 (8 ppm)] were monitored daily and remained within acceptable limits. Fish were hand-fed their respective diets twice daily at 09:00 and 16:00 to apparent satiety. Total daily feed consumption (g/fish on average) of fish in each tank was constant. Total fish weight in each tank was recorded every two weeks to minimize handling stress. Fish were weighed after a 24-h period of food deprivation. Almost 100% of water in tank was replaced daily. The feeding trial lasted for 56 days.

Sample collections and chemical analyses

At the beginning of the trial, 30 fish were euthanized with 140 ppm MS-222 (neutralized by an appropriate amount of $NaHCO_3$) for the analysis of whole body composition. At the end of the growth trial, 2 healthy fish per treatment were used for the analysis of whole body composition. Blood was collected with a hypodermic syringe from the caudal vein of 6 of conscious fish per

treatment group at 4 h post feeding. Blood samples were centrifuged at 8000 g and 4 ℃ for 2 min, and the supernatant fluid (serum) was stored at −80 ℃ until analysis. After the collection of blood samples, 6 fish per tank were euthanized to obtain visceral organs [including the stomach and intestine (without luminal contents), as well as liver, pancreas, and spleen)], peritoneal adipose tissue, and skeletal muscle. In addition, 6 fish with black skin syndrome were collected from different treatments to measure their body composition and morphometric parameters. All tissue samples were frozen in liquid nitrogen and stored at –80℃ for subsequent chemical analyses.

AAs in serum were measured by HPLC methods, as described previously (Collins et al. 2007). Briefly, the plasma sample (0.1 mL) was deproteinized with 0.1 mL of 1.5 mol/L $HClO_4$, followed by the addition of 0.05 mL of 2 M K_2CO_3. The mixture was centrifuged at 10,000 g for 1 min, and the supernatant fluid was analyzed for AAs by HPLC (Waters, Milford, MA, USA). AAs in diets were analyzed after acid hydrolysis as described by Hou et al. (2019). Crude protein (N × 6.25) was determined by the combustion method in Servi-Tech laboratories (Amarillo, TX, USA). Calcium and phosphorus were determined by optical spectrometry in Servi-Tech laboratories (Amarillo, TX, USA). Lipids were extracted from the samples with chloroform/methanol (2:1 v/v) according to the method of Folch et al. (1957).

Two healthy fish from each tank, as well as 6 fish with black skin syndrome were used for histologic analysis. Fresh tissues (~500 mg) were fixed with 4% paraformaldehyde (buffered to pH 7.2) for 24 h. The specimens were rinsed, dehydrated, and embedded in paraffin by Veterinary Medicine & Biomedical Sciences Histology Laboratory of Texas A&M University. Each sample was cut into two of 6-μm transverse sections with a rotary microtome. Sections from the intestine were stained with periodic acid-Schiff (PAS) staining method, and sections from the liver were

stained with the hematoxylin and eosin (H&E) staining method. The images were evaluated by using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axiocam HR digital camera. For the intestine, the radius of the organ, the height of the villus and mucosa, the width of the mucosa, submucosal thickness, and muscularis thickness were calculated by using the Axiovision 4.3 software of the instrument.

Statistical analysis

Results are expressed as means ± SEM. All data on body weight and metabolic profiles were analyzed by using one-way ANOVA and the Student Newman Keuls multiple comparison test. They were tested for homogeneity (the Levene's test) and normal distribution (the Kolmogorov-Smirnov test) before analysis. When the variances of data were not homogenous, log transformations were performed before ANOVA. All analyses were performed by using the SPSS package (version 19.0, SPSS Inc, Chicago, IL, USA). Probability values ≤ 0.05 were taken to indicate statistical significance.

Results

Feed composition and the costs of feedstuffs

The AA composition and the costs of different diets are summarized in Tables VI-2 and 3, respectively. Compared with the FM54 diet, the low fishmeal diets had much lower content of taurine and methionine. The content of dietary taurine decreased from 0.75 to 0.28 % (DM basis) and the content of dietary methionine decreased from 1.67 to 1.04 % (DM basis), as the dietary fishmeal protein content decreased from 54% to 5% (DM basis). The content of histidine, glycine, threonine and lysine in diets decreased slightly with decreasing the dietary fishmeal level. The price of different diets is listed in Table VI-3. Their costs decreased with decreasing dietary fishmeal levels. Note that the price of the FM5 diet was only about 43% and 30% of the FM54 diet

and a current commercial diet (AquaMax® Grower 400, Purina, MO), respectively.

Growth performance and feed utilization of fish

Data on the growth and feed utilization of fish fed the different diets for 8 weeks are summarized in Tables VI-4 and 5, respectively. At the end of the trial, the weight gain and final body weight were lower ($P < 0.05$) in fish fed the FM10 and FM5 diets than those fed the FM54 and FM30 diets ($P < 0.05$). Reducing the content of fishmeal protein from 54% to 5% reduced ($P < 0.05$) the growth of LMB by ~8%. At the end of the trial, no difference was observed in either the final body weight or weight gain among the FM54, FM30, and FM15 groups ($P > 0.05$). However, the body weight of fish in the FM15 group during days 14-42 was lower ($P < 0.05$) than that in the FM54 and FM30 groups.

Except for days 28 and 42, the feed conversion ratio (FCR) and protein efficiency ratio (PER) in fish fed the FM54 and F30 diets were improved over those in the FM5 group ($P < 0.05$). During days 42-56, the FCR and PER in the FM15 group were improved over those in the FM5 group ($P < 0.05$). At the end of the trial, the FCR or PER did not differ ($P > 0.05$) among fish fed the FM54, FM30, and FM15 diets. Both the FCR and the PER in these three groups of fish were improved ($P < 0.05$) in comparison with fish fed the FM10 and FM5 diets.

Data on the retention of dietary nitrogen, lipid, energy, phosphorus and calcium in the body are shown in Table VI-6. No difference was observed in the retention of dietary energy or lipids in the body among all the treatment groups ($P > 0.05$). At the end of the trial, fish fed the FM54, FM30 and FM15 diets had higher ($P < 0.05$) rates of retention of dietary nitrogen in the body, compared with those fed the FM10 and FM5 diets. The rates of retention of dietary phosphorus and calcium in the body were higher ($P < 0.05$) in fish fed the low fishmeal diets (FM15, FM10 and FM5) than those fed the FM54 and FM30 diets, which was associated with the reduced content

of dietary phosphorus in the low fishmeal feeds.

Body composition, morphometrical parameters

Data on the body composition and morphometric parameters of LMB fed different diets are summarized in Table VI-7. At the end of the 56-day trial, the content of water, protein, and lipids in the body of LMB was 71.0-71.6%, 16.9-17.3%, and 6.10-6.92 %, respectively. The content of water, protein, calcium or phosphorus in the whole body did not differ among the different dietary groups ($P > 0.05$). In contrast, the content of lipids in fish fed the FM10 and FM5 diets was higher ($P < 0.05$) than that in the FM54, FM30 and FM15 groups. The fish with black skin syndrome had lower values of the viscerosomatic index (VSI), intraperitoneal fat ratio (IPFR), and hepatosomatic index (HSI) than the healthy fish ($P < 0.05$). However, no difference was observed in VSI or HSI among the healthy fish in all the dietary groups ($P > 0.05$). Notably, fish fed the FM 30, FM15, and FM10 diets had a lower content of IPFR than those fed the FM54 and FM30 diets ($P < 0.05$).

Health and tissue histopathology of fish

The low fishmeal-based diets (FM15, FM10, and FM5) resulted in the occurrence of black skin syndrome after a 30-day period of feeding (Figs VI-1-5). At the end of the trial, about 13-15% of fish fed the low fishmeal diets (≤ 15% fishmeal protein) had black skin syndrome (Fig VI-1). As shown in Fig VI-2, the fish with the black skin syndrome had dark skin, different degrees of liver atrophy, pale livers with irregular staining, and cloudy eyes, and did not consume the feed well or completely failed to eat. Histological sections of the liver from the infected fish showed sinusoid disorganization. Generally, fish fed the high fishmeal diets had a better sinusoid structure than those fish fed the low fishmeal feeds (Fig VI-5). In fish fed the low fishmeal diets, intestinal enteritis occurred in the distal part of the gut intestine, which was characterized by a loose submucosal structure and the widening of the lamina propria of the intestinal mucosa (Fig VI-4).

Data on the intestinal morphology are shown in Table VI-8. The radius and the mucosal height of the proximal intestine in fish fed the FM5 diets were shorter ($P < 0.05$) than that in fish fed the FM54, FM30, and FM15 diets. The villus height of the proximal intestine in fish fed the FM10 and FM5 diets was shorter ($P < 0.05$) than that in fish fed the F54 and F30 diets. The mucosal width, submucosal thickness, and muscularis thickness of the proximal intestine in fish fed the FM10 and FM5 diets were decreased ($P < 0.05$) in comparison with the FM54 and FM30 groups. Except for the muscularis thickness, all the measured morphological variables in the proximal intestine did not differ ($P > 0.05$) between the FM10 and FM5 groups. The muscularis thickness of the proximal intestine in fish fed the FM5 diet was shorter ($P < 0.05$) than that in fish fed the FM10 diet. Values of all the measured morphological variables in the proximal intestine of fish with the black skin syndrome did not differ ($P > 0.05$) from those for fish fed the FM5 diet, but were all lower ($P < 0.05$) than fish fed the FM54, FM30 and FM15 diets. Similar results were obtained for the distal intestine of the fish.

Concentrations of AAs in serum

The concentrations of AAs in the serum of fish fed different diets are shown in Table VI-9. The concentrations of taurine, methionine, threonine, and histidine in serum were reduced ($P < 0.05$) in fish fed the FM5, FM10 and FM15 diets, compared with those fed the FM30 and FM54 diets. The concentrations of taurine, methionine, threonine, and histidine in the serum of fish fed the FM5 diet were 27%, 61%, 83%, and 57% of those in the FM54 group. Interestingly, fish fed the FM15, FM10 and FM5 diets had higher circulating levels of tyrosine than those in the FM54 and FM30 groups ($P < 0.05$). Fish fed the FM54 diet also had lower concentrations of tryptophan in serum than those fed the other diets ($P < 0.05$). The serum concentrations of branched-chain AAs, arginine, lysine, phenylalanine, ornithine, citrulline, alanine, glutamate, glutamine, glycine,

serine, aspartate, and asparagine were not affected by the dietary fishmeal level ($P > 0.05$).

Discussion

Low feed intake and poor growth performance often occur in carnivorous fish fed low fishmeal diets containing high levels of plant-source feedstuffs due to low palatability and the presence of anti-nutritional factors (Hardy and Shearer 1985; Francis et al. 2001; Kikuchi and Furuta 2009; Silva-Carrillo et al. 2012). For minimizing these negative effects of plant feedstuffs and controlling starch intake, high-protein feeds (~54% protein, DM basis) are commonly fed to LMB in the aquaculture industry. In the present study, the daily feed intake of fish (g/fish) was constant in all tanks by design to eliminate confounding factors that may preclude sound interpretations of results. Despite the same content of CP, lipids, and energy in all the diets, the growth performance of fish fed the FM54, FM 30 and FM15 diets was better than those fed the FM10 and FM5 diets. Such a difference was associated with substantial reductions in the content of both methionine and taurine in the low fishmeal diets. Of note, a recent study suggested that dietary fishmeal level for LMB could be reduced to 16% through the inclusion of poultry by-product meal plus soybean meal as alternative protein sources (Ren et al. 2018). Similarly, based on the growth performance and feed utilization, our results indicated that fishmeal protein can be reduced from 54% to 15% (DM basis) by soybean meal and poultry by-product meal protein without additional AA supplementation.

Much evidence shows that low fishmeal diets hinder the growth and feed utilization of carnivorous fish, partly because plant-source proteins have an imbalance of AAs relative to nutritional needs (Li et al. 2011; Li and Wu 2020). In this study, the deficiency of methionine and taurine (Table VI-2) in the FM10 and FM5 diets may be the main reason for the poor growth of LMB. Previous studies have shown that dietary supplementation with one or more limiting AAs

can improve the growth performance of fish fed low-fishmeal diets (Venou et al. 2006; Kader et al. 2010). There is also evidence that dietary supplementation with methionine and lysine is necessary for the complete replacement of fishmeal with alternative protein sources in cobia (Salze et al. 2010) and *Oncorhynchus mykiss* (Yamamoto et al. 2005). Clearly, a proper balance among dietary AAs is crucial for optimizing the retention of dietary AAs as proteins in the body (Aragão et al. 2004; Saavedra et al. 2009). This is supported by our metabolic data showing that the serum concentrations of methionine, histidine, threonine and taurine were reduced in LMB fed the low fishmeal diets. There is also a possibility that fishmeal may contain functional nutrients (e.g., polyamines and small peptides) that can improve intestinal and whole-body health, as well as the growth of fish, including LMB (Li and Wu 2020).

Partial or even total replacement of fishmeal by alternative protein sources has been achieved successfully in several carnivorous species (Daniel 2018). In the present study, low-fishmeal diets (≤15% protein, DM basis) can be recommended for practical production because the cost of low fishmeal diets was only ~40% of that for the whole fishmeal diet (Table VI-3). Moreover, low-fishmeal diets are also more environmentally friendly because of their low phosphorus content but high retention rates in the body. However, increasing plant proteins such as soybean meal in the diets of carnivorous fish may compromise their immune system and induce intestinal inflammation (Daniel 2018; Hardy 2010). In this study, we discovered that LMB fed diets containing ≤ 15% fishmeal protein (DM basis) exhibited the black skin syndrome, which was characterized by the darkening of the skin along with damages in the eyes, intestine and liver (Fig VI-2). The presence of this disorder started from day 30, and about 13-15% of the fish fed the FM15, FM10 and FM5 diets had this problem by day 56 of the feeding trial. Fish with the black skin syndrome often did not consume the feed provided, possibly due to vision dysfunction, and had very low rates of

growth. Because of reduced feed intake, those fish had lower values of the VSI and IPFR when compared with the healthy fish due to the mobilization of body fats. Most of the black-skin fish did not die immediately until they were injured as a result of aggression by other fish in the same tank or by bacterial infection. Consequently, the production of LMB would be severely hindered by this previously unrecognized metabolic disorder on farms.

Until now, the black-skin syndrome of nutritional origin has not been reported for LMB, and its pathogenesis is unknown. A possible reason may be a lack of one or more AAs in the alternative proteins because these nutrients are vital to the growth, development, and health of animals, including fish (Li et al. 2007 and 2009). In support of this view, we found that the concentrations of taurine, methionine, threonine, and histidine in the serum of LMB were substantially reduced when the fish were fed the low fishmeal diets, as noted previously. The beneficial functions of methionine on immune and antioxidant defenses have been reported in fish (Elmada et al. 2016; Kuang et al. 2012; Machado et al. 2015). Taurine also plays an important role in fat digestion, antioxidative defense, cellular osmoregulation, as well as the development of visual, neural and muscular systems (Li et al. 2009). At present, it is unknown whether or not LMB can synthesize taurine. Interestingly, Takeuchi (2014) reported that pigmentation was altered in seawater fish fed a diet containing high levels of taurine. To our knowledge, such a phenomenon has not been reported for LMB.

The liver of fish shows a high variability in both composition and size among individuals and species, and is considered as a primary organ for the metabolic control of growth and nutritional traits (including fillet quality and quantity) (Brusle et al. 1996). Normally, the liver shows reddish brown color due to its rich vascularity (as shown in Fig VI-2) and its high content of iron, which indicates that the animals are in good nutritional status and good health (Bruslé et al. 1996).

However, the liver of fish with black-skin syndrome had pale and yellowish color with irregular staining, indicating that the liver was damaged. Liver size (HSI) is also highly sensitive to nutritional status in many fishes, and its irregular size can be an important indicator for low-quality food. Consistently, the HSI was much lower in fish with black-skin syndrome (0.9%) than that in the healthy fish (1.25-1.43%). Histological analysis of the digestive system (such as the intestine and liver) is considered a good indicator of the nutritional and health status of fish (Rašković et al. 2011; Torrecillas et al. 2017). In this study, sinusoid disorganization was clearly observed in the liver of the fish fed the low-fishmeal diets. Sinusoids receive blood directly from portal vein radicles with which they are contiguous, along with hepatic artery radicles (Wilkins et al. 2013; Akiyoshi and Inoue 2004). A good structure of hepatic sinusoids is essential to proper hepatic function (Akiyoshi and Inoue 2004). The sinusoid disorganization leads to the poor vascularity and, consequently, a pale and yellowish liver due to impaired blood flow. Collectively, our results indicated that low-fish meal diets damaged the structure and function of the liver, possibly resulting in the black skin syndrome in LMB.

Another important observation from the present study is that soybean meal and poultry by-product meal substitution altered the intestinal morphology of LMB. The radius of the intestine, the heights of the villus and mucosa, the thickness of submucosal and muscularis in both the proximal and the distal segments of the gut decreased with decreasing levels of fishmeal in the diet, which may be related to deficiencies of taurine and methionine. Of note, dietary supplementation with taurine to soybean meal-based diets could increase the length of villi folds, reduce the number of vacuoles, and increase the number of goblet cells in European sea bass (Rimoldi et al. 2016). Decreases in the length of villi and the number of goblet cells were also observed in turbot (*Scophthalmus maximus* L.) fed methionine-deficient diets (Gao et al. 2019).

Moreover, several antinutritional factors present in soybean meal may cause changes in the intestinal morphology of fish (Miao et al. 2018). For example, increasing the dietary saponin level from 0.0 to 3.2 and 6.4 g.kg diet reduced the height of the intestinal villi and the number of intestinal goblets in juvenile Japanese flounder (*Paralichthys olivaceus*) (Chen et al. 2011). It should be noted that a low rate of growth in adult or juvenile fish is generally related to the dysfunction of the intestinal system (Johnston 2001). This is also in line with the poor growth performance of LMB fed low-fishmeal diets. Polyamines, which are abundant in fishmeal (Li and Wu 2020), may play an important role in the intestinal health of fish as in land animals (Wu 2018).

Another salient and important finding from the present work is that the concentrations of tyrosine and tryptophan in the serum of LMB fed the low fishmeal diets (≤ 15% protein, DM basis) were much higher than those in fish fed the FM54 diet. In mammals, the liver and the kidneys are major organs for converting phenylalanine into tyrosine by phenylalanine hydroxylase, and tyrosine is completely oxidized in the liver (Wu 2013b). As a result, the increase of tyrosine can be observed in animals with a dysfunctional liver (Ninomiya et al. 1999; Dashti et al. 1994). Likewise, an increase in plasma tyrosine concentrations occurred in rats after a single dose of hepatotoxins to damage the liver (Clayton et al. 2007). Thus, plasma tyrosine concentration is a potentially useful biomarker of hepatoxicity for toxicological screening and liver damage. Furthermore, higher levels of tryptophan in plasma and cerebrospinal fluid are also characteristically reported for chronic liver failure in humans and experimental animals (Laviano et al. 1997; Salerno et al. 1984). Because the content of tyrosine, tryptophan or phenylalanine was similar among the different experimental diets (Table VI-2), the elevated concentrations of both tyrosine and tryptophan in the serum of LMB fed the low fishmeal diets may be indicative of hepatic dysfunction and hepatoxicity brought about by poultry byproduct meal or soybean meal.

Moreover, excess tyrosine (hypertyrosinemia) and tryptophan (hypertryptophanemia) in serum are toxic to animals. For example, tyrosinemia induces eye and skin lesions, which can be treated with a low-tyrosine and low-phenylalanine diet (Goldsmith and Reed 1976). Similarly, Gipson and Anderson (1977) reported that feeding rats a diet high in tyrosine induced a reproducible focal corneal epithelial lesion. The relationship between high levels of the aromatic AAs (tyrosine and tryptophan) in serum and black skin syndrome in LMB should be further addressed.

Our results indicated that 15% fishmeal protein (DM basis) in the diet containing poultry by-product meal and soybean meal was sufficient for the growth of juvenile LMB. However, the fish fed the FM15, FM10 and FM5 diets developed the hitherto unrecognized black skin syndrome possibly due to deficiencies of methionine and taurine. The serum concentrations of taurine and methionine, along with threonine, and histidine, were much lower in fish fed the low fishmeal diets than those in fish fed higher fishmeal (30-54% protein, DM basis) diets. Additionally, low fishmeal levels damaged the liver of LMB, leading to elevated concentrations of both tyrosine and tryptophan in serum.

Table VI-1 Composition and proximate analyses of the experimental diets

Ingredient [% of dry matter (DM)]	FM54	FM30	FM15	FM10	FM5
Fish meal menhaden[1]	78.37	43.54	21.77	14.51	7.26
Poultry by-product meal (PBM)[2]	---	22.13	35.97	40.58	45.19
Soybean meal (SBM)[3]	---	17.50	28.44	32.08	35.73
Poultry fat[4]	5.96	2.20	---	---	---
Fish oil menhaden[5]	---	2.84	4.49	4.30	3.69
Dextrinized starch	5.00	4.60	4.40	4.40	4.30
Vitamin premix[7]	1.00	1.00	1.00	1.00	1.00
Mineral premix[8]	2.00	2.00	2.00	2.00	2.00
Cellulose[9]	7.53	3.70	1.23	0.35	0.00
Choline chloride	0.14	0.14	0.14	0.14	0.14
Composition (DM basis)*					
Crude protein (%)	54.0	54.0	54.0	54.0	54.0
Crude lipids (%)	13.0	13.0	13.0	13.0	13.0
Phosphors (%)	2.52	2.21	1.79	1.75	1.44
Calcium (%)	4.26	3.29	2.59	2.11	1.98
Energy (kJ/g of DM)	18.2	18.2	18.2	18.2	18.2

* The content of dry matter (DM) in the diets (as-fed basis) was 96.0%.

[1] Omega Fish Meal (Corporate Headquarters of Omega Protein, Houston, Texas).

[2] Poultry by-product meal (Tyson Foods, AR, USA).

[3] 48% Soybean meal (Hendricks Feed & Seed, IA, USA).

[4] Chicken fat (Tyson Foods, AR, USA).

[5] Fish oil (Paragon, IL, USA).

[6] Maltodextrin (Baolingbao Biology, Shangdong, China).

[7] Vitamin premix (g/kg): vitamin A, 2.31; vitamin D_3, 2.02; vitamin E, 20.00; vitamin K_3, 1.2; vitamin C, 30.00; vitamin B_5, 10.87; inositol, 15.00; niacin, 14.00; vitamin B_6, 3.04; vitamin B_2, 3.00; vitamin B_1, 3.26; biotin, 0.15; folic acid, 0.6; vitamin B_{12}, 0.02; cellulose, 894.53.

[8] Mineral premix (g/kg): NaCl, 181.94; $MgSO_4.7H_2O$, 293.33; $FeSO_4.7H_2O$, 11.11; $AlCl_3.6H_2O$, 0.33; KI, 0.33; $CuSO_4.5H_2O$, 1.11; $MnSO_4$, 2.33; $CoCl.6H_2O$, 0.43; $ZnSO_4.7H_2O$, 9.04; Na_2SeO_3, 0.33; cellulose, 500.00.

[9] Microcrystaline cellulose 102 (Blue Diamond Growers, California, USA).

Table VI-2 Composition of amino acids in experimental diets (g/100 g of dry matter)[1]

Amino acid	FM54	FM30	FM15	FM10	FM5
Asp	2.83	2.85	2.86	2.87	2.88
Asn	1.91	1.92	1.93	1.94	1.93
Glu	4.48	4.20	4.01	3.96	3.95
Gln	3.02	3.00	2.98	2.96	2.96
Ser	2.01	1.98	1.97	1.96	1.94
His	1.15	1.10	1.05	1.04	1.05
Gly	2.85	3.13	3.29	3.35	3.36
Thr	1.96	1.90	1.87	1.86	1.85
Arg	3.57	3.43	3.32	3.28	3.25
Ala	3.06	2.77	2.58	2.52	2.47
Tyr	1.61	1.60	1.58	1.57	1.55
Trp	0.54	0.55	0.55	0.54	0.55
Met	1.52	1.21	1.03	0.98	0.92
Val	2.30	2.21	2.15	2.08	2.06
Phe	1.93	1.93	1.92	1.94	1.93
Ile	2.04	1.95	1.88	1.87	1.86
Leu	3.45	3.36	3.31	3.29	3.28
Lys	3.68	3.26	2.97	2.90	2.89
Pro	2.74	2.91	2.97	2.99	3.01
Hyp	1.16	1.14	1.12	1.12	1.13
Taurine	0.70	0.49	0.34	0.30	0.27
Cysteine	0.50	0.60	0.67	0.69	0.69

[1]Values are means for 3 feed samples. The content of proteinogenic amino acids in diets (0.25 g) was determined by HPLC (Li and Wu 2020) after hydrolysis in 6 M HCl at 110 ^{o}C for 24 h and, then calculated on the basis of the molecular weights of residue amino acids. The content of taurine in diets (0.25 g) was determined by HPLC (Li and Wu 2020) after homogenization in 2 ml of 1.5 M $HClO_4$ and, then calculated on the basis of its intact molecular weight.

Table VI-3 Costs of different diets used for this experiment.

Diet	Price ($/kg)	Percentage of the price of the unsubstituted fish meal diet (54% protein, DM basis)	Percentage of the price of commercial diet
FM54	1.4	100	70
FM30	1.0	71	50
FM15	0.8	57	40
FM10	0.7	50	35
FM5	0.6	43	30
Commercial diet*	2.0	143	100

* AquaMax® Grower 400 (Purina, MO, USA).

Table VI-4 Growth performance of LMB fed diets with different fishmeal levels[1].

	Body weight (BW; g/fish)					Weight gain over initial BW (%)			
	Initial	Day 14	Day 28	Day 42	Day 56	Days 0-14	Days 0-28	Days 0-42	Days 0-56
FM54	4.93	12.0^{a}	18.6^{a}	25.5^{a}	35.0^{a}	143.4^{a}	277.8^{a}	417.2^{a}	610.2^{a}
FM30	4.95	11.8^{a}	18.4^{a}	25.7^{a}	34.9^{a}	137.9^{a}	270.5^{ab}	418.3^{a}	605.1^{a}
FM15	4.93	11.3^{b}	17.8^{b}	24.8^{b}	33.4^{ab}	129.5^{b}	261.2^{bc}	404.0^{ab}	578.5^{ab}
FM10	4.94	11.0^{b}	17.9^{b}	24.2^{c}	32.3^{b}	123.1^{b}	263.5^{bc}	389.1^{b}	554.1^{b}
FM5	4.90	11.0^{b}	17.2^{c}	24.4^{bc}	32.2^{b}	124.2^{b}	251.9^{c}	397.8^{b}	556.3^{b}
SEM	0.02	0.10	0.11	0.15	0.33	1.97	2.48	3.22	6.56
P values		<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.003	0.001

[1] Values are means with pooled SEM for 4 tanks of fish per treatment group.
[a-c]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table VI-5 Feed utilization of LMB fed diets with different fishmeal levels[1].

Diet	Feed conversion ratio (feed DM intake/body weight gain)					Protein efficiency ratio (body weight gain/protein intake)				
	Days 0-14	Days 14-28	Days 28-42	Days 42-56	Days 0-56	Days 0-14	Days 14-28	Days 28-42	Days 42-56	Days 0-56
FM54	0.62[b]	0.91	0.92[b]	0.93[b]	0.85[b]	2.91[a]	2.04	1.91[a]	2.00[a]	2.18[a]
FM30	0.64[b]	0.91	0.87[b]	0.95[b]	0.85[b]	2.76[b]	2.03	2.03[a]	1.97[a]	2.18[a]
FM15	0.69[a]	0.93	0.90[b]	0.97[b]	0.88[b]	2.58[c]	2.00	1.95[a]	1.92[a]	2.11[a]
FM10	0.72[a]	0.87	1.02[a]	1.04[ab]	0.92[a]	2.46[c]	2.14	1.72[b]	1.78[b]	2.01[b]
FM5	0.72[a]	0.97	0.89[b]	1.11[a]	0.93[a]	2.46[c]	1.93	1.99[a]	1.67[b]	1.99[b]
SEM	0.01	0.01	0.02	0.02	0.01	0.04	0.02	0.03	0.04	0.02
P values	<0.001	0.112	0.008	0.028	< 0.001	<0.001	0.088	0.015	0.015	<0.001

[1] Values are means with pooled SEM for 4 tanks of fish per treatment group.
[a-c]: Within a column, means not sharing the same superscript are different ($P < 0.05$). DM, dry matter.

Table VI-6 The retention (or productive value, %) of dietary energy and nutrients in the body of LMB fed diets with different fishmeal levels[1].

Diet	Nitrogen	Lipids	Energy	Phosphorus	Calcium
FM54	37.0[a]	58.2	45.7	33.9[b]	32.0[d]
FM30	37.1[a]	58.3	45.7	38.0[b]	41.5[c]
FM15	35.9[a]	58.1	42.8	44.1[a]	52.2[b]
FM10	34.2[b]	59.1	43.6	44.3[a]	61.5[a]
FM5	343.8[b]	62.1	43.5	49.1[a]	58.9[ab]
SEM	0.36	0.73	0.42	1.4	2.7
P values	< 0.001	0.382	0.063	<0.001	<0.001

[1] Values are means with pooled SEM for 4 tanks of fish per treatment group.
[a-c]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table VI-7 The body composition and morphometrical parameters (% of wet body) in LMB fed diets with different fishmeal levels.

Diet	Body composition[1]					Morphometrical parameters[2]		
	Water	Protein	Lipids	Calcium	Phosphorus	VSI	HSI	IPFR
FM54	71.0	16.9	6.10[c]	1.17	0.733	8.75[a]	1.43[a]	2.96[a]
FM30	71.4	17.1	6.11[c]	1.17	0.720	8.56[a]	1.48[a]	2.88[a]
FM15	71.6	16.4	6.26[c]	1.17	0.703	7.99[a]	1.28[a]	2.46[b]
FM10	71.9	17.3	6.54[b]	1.19	0.710	8.10[a]	1.37[a]	2.51[b]
FM5	71.5	17.1	6.92[a]	1.11	0.678	8.03[a]	1.25[a]	2.58[b]
Black fish						6.48[b]	0.90[b]	1.73[c]
SEM	0.13	0.11	0.07	0.01	0.02	0.12	0.04	0.07
P values	0.242	0.092	<0.001	0.654	0.414	<0.001	<0.001	<0.001

[1] Values are means with pooled SEM for 4 tanks of fish per treatment group.
[2] Values are means with pooled SEM for 12 fish per treatment group. Viscerosomatic index (VSI), intraperitoneal fat ratio (IPFR), and hepatosomatic index (HSI).
[a-c]: Within a column, means not sharing the same superscript are different ($P < 0.05$).

Table VI-8 Morphometrical measurements of proximal and distal intestines in LMB fed diets with different fishmeal levels[1]

	FM 54	FM 30	FM 15	FM 10	FM 5	Black fish	SEM	P values
Proximal intestine (PI)								
Radius of the organ (µm)	928.3^{a}	866.2^{a}	873.5^{a}	858.3^{ab}	769.6^{bc}	696.7^{c}	22.7	0.021
Height of the villus (µm)	551.7^{a}	547.7^{a}	509.9^{ab}	470.7^{bc}	436.3^{c}	433.6^{c}	12.5	0.001
Height of the mucosa (µm)	602.9^{a}	595.9^{a}	590.8^{ab}	560.3^{bc}	527.7^{cd}	492.3^{d}	11.5	0.011
Width of the mucosa (µm)	105.3^{a}	107.1^{a}	72.8^{b}	73.6^{b}	65.3^{b}	64.4^{b}	4.18	0.003
Submucosal thickness (µm)	30.7^{a}	26.7^{ab}	25.5^{ab}	23.3^{b}	21.9^{b}	24.6^{ab}	0.86	0.031
Muscularis thickness (µm)	54.2^{a}	57.2^{a}	48.6^{ab}	41.3^{bc}	32.4^{d}	36.9^{cd}	2.27	<0.001
Distal intestine (DI)								
Radius of the organ (µm)	767.5^{a}	777.3^{a}	760.1^{a}	734.4^{ab}	699.4^{bc}	660.8^{c}	12.4	0.024
Height of the villus (µm)	467.7^{a}	465.3^{a}	457.5^{a}	432.3^{a}	394.2^{b}	351.5^{c}	9.93	0.005
Height of the mucosa (µm)	553.4^{a}	532.6^{a}	514.5^{a}	503.5^{a}	420.8^{b}	420.5^{b}	11.8	<0.001
Width of the mucosa (µm)	98.1^{a}	99.2^{a}	96.6^{a}	88.3^{b}	94.0^{ab}	77.6^{c}	1.76	<0.001
Submucosal thickness (µm)	22.9^{b}	22.0^{b}	21.3^{b}	17.2^{c}	15.6^{c}	30.6^{a}	1.05	<0.001
Muscularis thickness (µm)	76.1^{a}	68.7^{ab}	63.0^{bc}	65.1^{bc}	46.2^{d}	58.5^{c}	2.21	<0.001

[1] Values are means with pooled SEM for 4 tanks of fish per treatment group. There were 6 fish per tank.
$^{a\text{-}c}$: Within a row, values not sharing the same superscript are different ($P < 0.05$).

Table VI-9 Concentrations (μM) of free amino acids in the serum of juvenile LMB at 4 h after consuming diets containing different fishmeal levels[1]

	FM54	FM30	FM15	FM10	FM5	SEM	*P* value
Asp	30.6	34.8	34.4	34.1	35.7	1.44	0.151
Glu	54.2	56.7	52.6	57.2	57.3	1.65	0.885
Asn	318	340	327	337	365	11.2	0.346
Ser	591	593	575	574	635	14.9	0.467
Gln	366	408	393	360	371	8.07	0.218
His	255[a]	219[b]	172[c]	146[d]	144[d]	9.19	< 0.001
Gly	1300	1243	1261	1107	1258	38.9	0.609
Thr	512[a]	519[a]	425[b]	403[b]	424[b]	14.1	0.002
Cit	208	200	187	193	181	5.64	0.302
Arg	38.5	41.3	40.1	40.1	39.6	2.35	0.846
β-Ala	31.3	27.6	26.2	32.5	31.5	1.21	0.215
Taurine	1505[a]	1231[b]	656[c]	550[d]	403[e]	60.2	< 0.001
Ala	943	1114	1045	1014	1018	30.2	0.529
Tyr	152[d]	228[c]	274[b]	288[b]	383[a]	14.9	< 0.001
Trp	53.1[b]	69.4[a]	73.0[a]	75.2[a]	79.1[a]	2.20	0.003
Met	209[a]	210[a]	127[b]	120[b]	128[b]	9.22	< 0.001
Val	843	928	912	883	875	24.8	0.571
Phe	210	246	244	230	241	7.78	0.124
Ile	279	321	304	263	284	7.16	0.085
Leu	506	558	561	474	525	19.7	0.456
Orn	215	244	201	200	213	6.51	0.730
Lys	299	320	303	281	295	14.0	0.220

[1] Values are means with pooled SEM for 6 fish per dietary group.
[a-c]: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

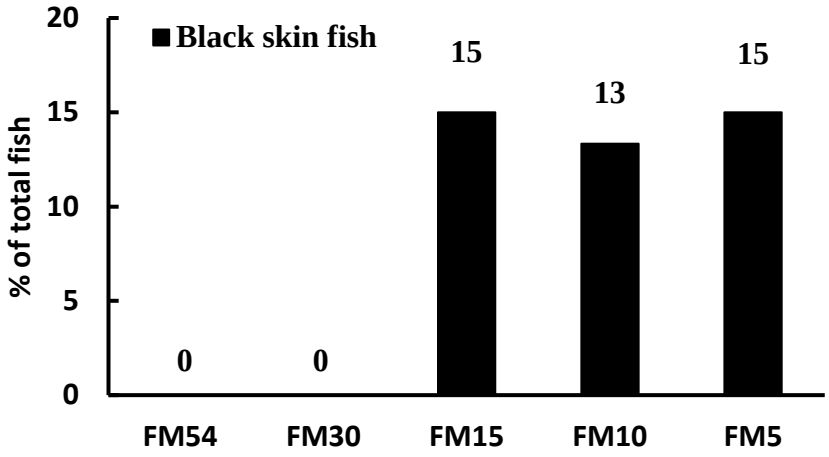


Figure VI-1 The incidence of black skin syndrome in fish fed diets with different fishmeal levels. Some of the fish (13-15%) fed the FM15, FM30, and FM5 diets developed black skin syndrome, and they did not eat feeds well or failed to consume any feeds. No black skin fish were observed in the FM54 and FM30 groups. No fish was dead in each treatment group at the end of the 8-week experiment.

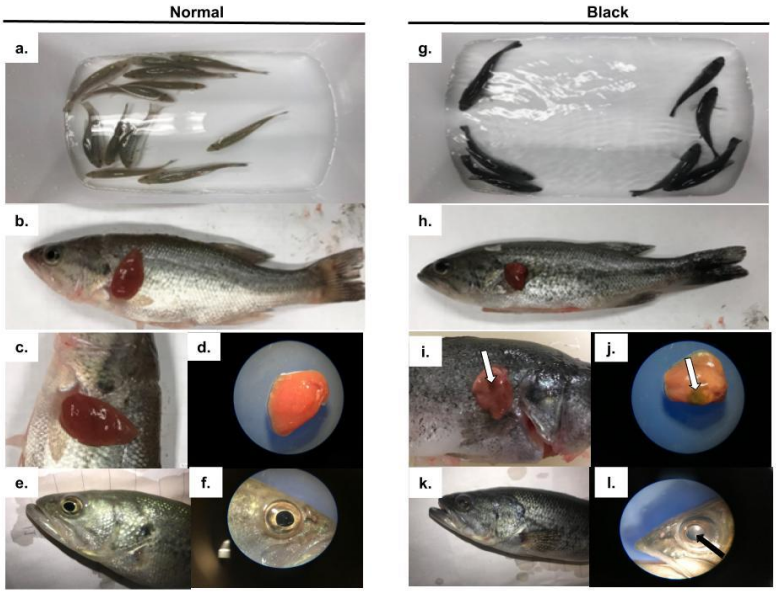


Figure VI-2 The symptom of black skin in fish fed low fishmeal (⩽15% protein, DM basis) diets. a-f. Fish with normal body color, healthy liver and eyes, and they consumed feeds well during the experiment. g-i. Fish with black skin had the atrophy of liver. The liver of fish with black skin syndrome had pale color with irregular staining (white arrow). All of these fish had different degrees of cloudy eye problem (black arrow).

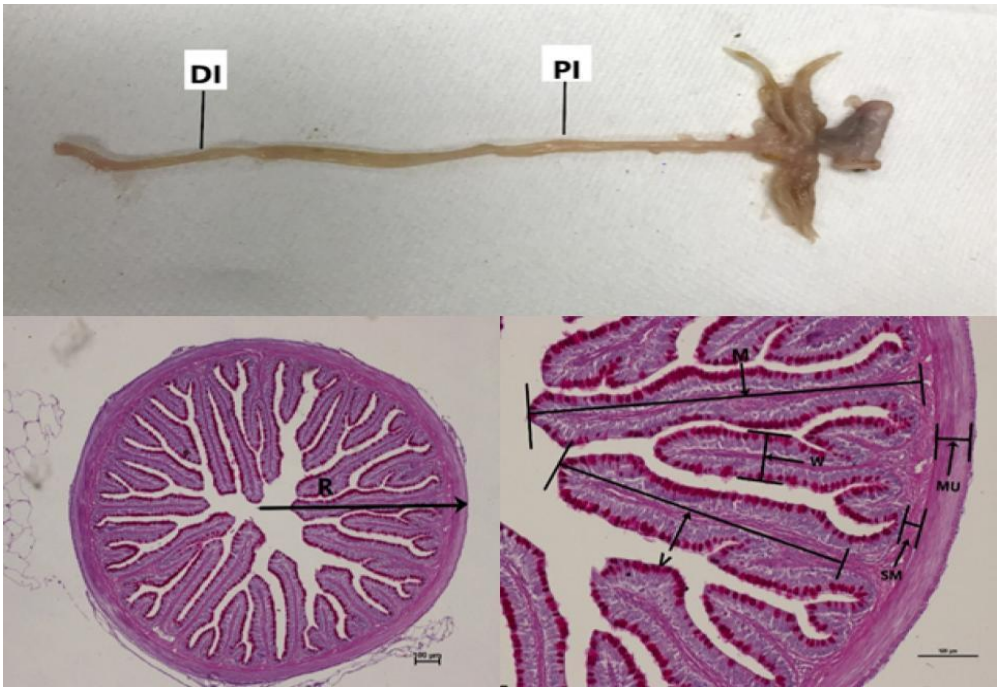


Figure VI-3 The sample sites and morphometrical measurements of the proximal intestine (PI) and distal intestine (DI) in experimental fish. R, radius of the organ; V, height of the villus; M, height of the intestinal mucosa; W, width of the intestinal mucosa; SM, thickness of the submucosa; MU, thickness of the muscularis.

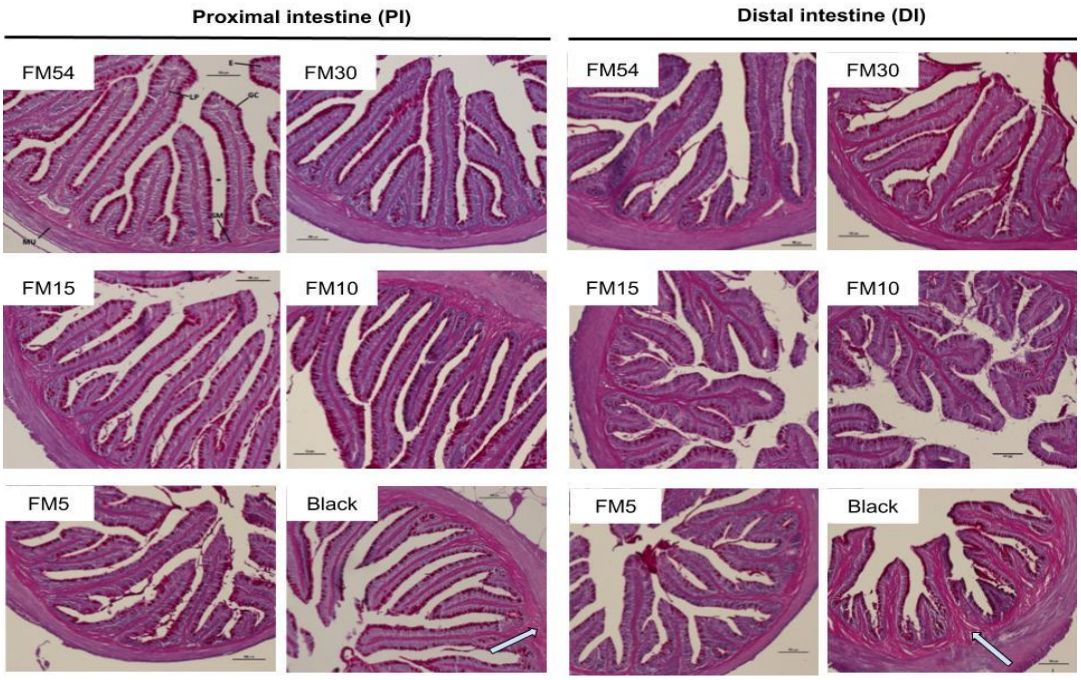


Figure VI-4 Histological analysis of the proximal intestine (PI) and distal intestine (DI) of LMB fed diets with different levels of fishmeal. The tissues were stained with PAS. Arrow: intestinal enteritis was observed in the intestine, which is characterized by the loose submucosal structure and widening of the lamina propria of the mucosa.

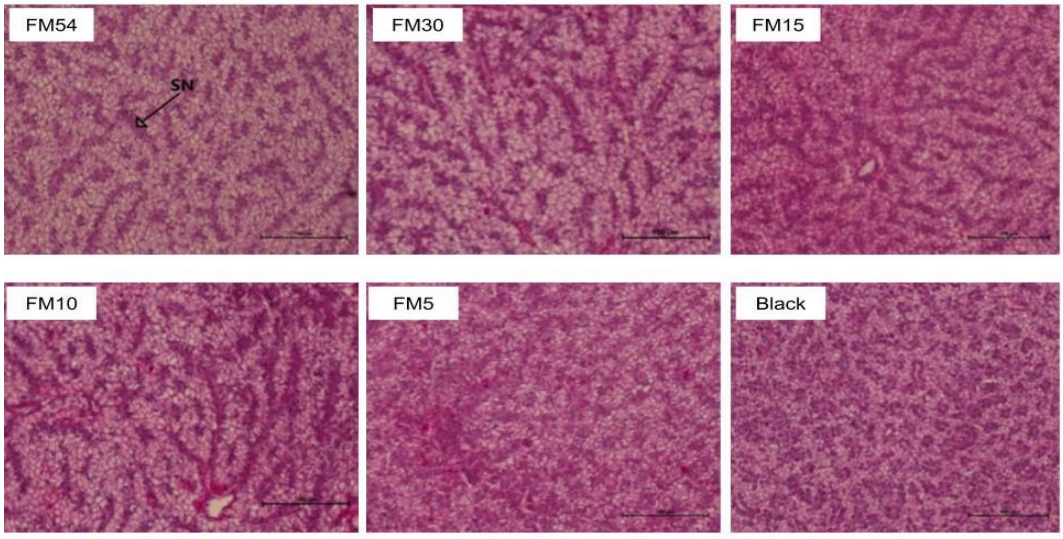


Figure VI-5 Histological analysis of the liver of LMB stained with H&E. Note: Healthy fish had a clear sinus (SN) structure which could not be clearly observed in fish fed the FM5 diet and in fish with black skin (Black).

CHAPTER VII

EFFECTS OF REPLACING FISHMEAL BY A MIX OF ALTERNATIVE PROTEIN FEEDSTUFFS ALONG WITH TAURINE OR METHIONINE ON THE GROWTH, FEED UTILIZATION, AND HEALTH OF LMB (*MICROPTERUS SALMOIDES*)

Abstract Fishmeal has long been a staple protein feedstuff for fish, but its global shortage and high price have prompted its replacement with alternative sustainable sources. In this experiment involving largemouth bass (a carnivorous fish), a new mixture of 53.5% protein feedstuffs (45% poultry byproduct meal, 30% soybean meal, 15% blood meal, and 10% krill shrimp meal) was added to low fishmeal (14.5%) diets along with 0.5% taurine, 0.5% methionine or both (dry matter basis). The positive control diet contained 65.3% fishmeal (45% fishmeal protein on dry matter basis). All diets contained the same levels of digestible crude protein. Fish were fed to satiety twice daily. Compared with the unsupplemented low-fishmeal group, supplementing either 0.5% methionine or 0.5% methionine plus 0.5% taurine to the low-fishmeal diet improved ($P < 0.05$) the growth, feed utilization, retention of dietary protein and lipids, and health of largemouth bass, reduced ($P < 0.05$) the occurrence of black skin syndrome from 40% to 10%. Histological sections of eyes from fish with black skin syndrome showed retina degeneration, liver damage, and enteritis in intestine. Compared with methionine supplementation, supplementing 0.5% taurine alone to the low-fishmeal diet had lesser beneficial effects ($P < 0.05$) on ameliorating the black skin syndrome. These results indicated that: (a) the basal low-fishmeal diet was inadequate in methionine or taurine; and (b) dietary supplementation with methionine was an effective

method to improve the growth performance, feed efficiency, and health of LMB. Further studies are warranted to understand the pathogenesis of the black skin syndrome in LMB.

Introduction

Aquaculture is the fastest-growing agricultural enterprise to provide high-quality animal protein for human consumption (Huang et al. 2017; Li et al. 2019). About 70% of the global aquaculture (excluding aquatic plants) relies on commercial compound feeds that is produced by mixing feed ingredients, including fishmeal as the main staple protein feedstuff (Béné et al. 2016). During the past 20 years, considerable progress has been made towards replacing portions of fishmeal in aquafeeds with alternative protein sources. Many previous studies have contributed to an impressive reduction in the average inclusion of fishmeal in compounded feeds from 1995 to 2010 for major groups of farmed species (Hardy 2010; Olsen and Hasan 2012). In some fish species, results even supported a complete replacement of fishmeal by plant protein without affecting their growth performance (Daniel 2018; Hua et al. 2019). Most of these studies have drawn such a conclusion based on the results of growth, feed intake, and nutrient utilization. However, little is known about the impact of low fishmeal diets on the health of fish.

Diets have profound effects on stress tolerance and health. Therefore, fish fed inadequate amounts of nutrients, particularly AAs, have a high susceptibility to disease and the appearance of deficiency signs, including altered behavior and pathological changes (Oliva-Teles 2012; Wu 2020a). Moreover, metabolic disorders (including metabolic diseases) in animals can occur due to one or more of the following reasons: low-quality diet; inadequate or excessive intake of nutrients; impairments in digestion, absorption, utilization, or storage of nutrients; imbalances and antagonisms among nutrients; excessive

excretion of nutrients; and toxins in the diet (Wu 2020a). Diets with little or no fishmeal could lead to negative impact on fish health, because plant-source feedstuffs contain lower content of methionine than fishmeal and lack taurine (Li and Wu 2020). Evidence shows that some AAs and their metabolites are important regulators of key metabolic pathways that are necessary for maintenance, growth, immunity, behavior, as well as resistance to environmental stressors and pathogenic organisms in various fishes (Li et al. 2009). For example, high levels of dietary soybean products and terrestrial meals affect intestinal integrity in Atlantic cod (*Gadus morhua* L.; Olsen et al. 2007) and European sea bass (*Dicentrarchus labrax*; Torrecillas et al. 2017), respectively. The symptoms of vascular congestion and multifocal and random mononuclear infiltration aggregates were observed in totoaba juveniles (*Totoaba macdonaldi*) fed diets with fishmeal replacement by soy protein concentrate (López et al. 2015).

Recently, pellet diets have been developed for largemouth bass (*Micropterus salmoides;* LMB, a carnivorous fish) that is native to North America. However, the requirement of LMB for dietary protein is generally ≥ 45% (Huang et al. 2017; Ding et al. 2019). A previous study revealed that dietary fishmeal level could be reduced to 16% for LMB by the inclusion of poultry by-product meal and soybean meal based on fish growth and feed utilization (Ren et al. 2018). However, we recently discovered that LMB fed diets with low fishmeal develop black skin syndrome (characterized by dark skin, as well damages in the eyes, liver and intestine) even their growth performance and feed utilization are not significantly different (Chapter VI). Thus, this study was designed to evaluate the effects of replacing fishmeal by a mix of alternative protein feedstuffs along with taurine or methionine supplementation on the growth, feed utilization, and health of LMB. The

protein sources used were poultry by-product meal, soybean meal, blood meal, and krill shrimp meal.

Materials and Methods

Experimental diets

A mix of protein feedstuffs (fishmeal, soybean meal, blood meal, and krill shrimp meal) with or without supplementation with taurine or methionine, was used to reduce the dietary fishmeal content from 65.3% to 14.5% (DM basis; a 78% replacement). Five experimental diets were designed as the whole fishmeal group (FM), low fishmeal group (MP), low fishmeal supplemented with 0.5% taurine (MP + Tau), low fishmeal supplemented with 0.5% methionine (MP + Met), and low fish meal supplemented with 0.5% methionine plus 0.5% taurine (MP + Met & Tau). The proportions of soybean meal and fishmeal in the diets were based on the studies of Ren et al. (2018).

Blood meal, which is rich in histidine (Li et al. 2011), was used to balance histidine content in the low fishmeal diets, because we recently found that serum histidine concentration was reduced in fish fed the low fishmeal diets containing large amounts of soybean meal and poultry by-product meal than in fish fed the whole fishmeal diet (Chapter VI). Similarly, methionine and/or taurine were added to the low fishmeal diets (Table VII-1), because (a) we recently found that the concentrations of both methionine and taurine were reduced in fish fed the low fishmeal diets containing large amounts of soybean meal and poultry by-product meal than in fish fed the whole fishmeal diet (Chapter VI); and (b) there are reports that a deficiency of methionine and taurine in plant-source feedstuffs can be a limiting factor for successful fishmeal replacement (Ai et al. 2005; Figueiredo-Silva et al. 2015; Nunes et al. 2014; Oliva-Teles 2012). Krill shrimp meal

was added to the low fishmeal diets as a strategy to stimulate the feed intake of LMB, as previously reported for some fish species (Shimizu et al., 1990; Tibbetts et al., 2011). In our preliminary study, we found that juvenile LMB accepted very well such low-fishmeal diets and had good growth performance. All diets were formulated to contain the same amount of true protein (40.1%) and lipid (9.7%) levels. The ingredients and proximate composition of diets are shown in Tables VII-1 and 2. All dry ingredients were thoroughly mixed, followed by the addition of oil and water to form a moist dough. The experimental diets were produced by a screw extruder (Big Bite Meat Grinder, West Chester, OH) and oven-dried at 45 ^{o}C. All feeds were stored at -20 ℃ until use within 4 weeks.

Experimental animals and their rearing system

Juvenile LMB were obtained from a commercial fish farm (Larry's Fish Farm, Giddings, TX, USA) and housed in the Kleberg Center of Texas A&M University, as previously described (Jia et al. 2017). The photoperiod of the housing facility was maintained for 14 h per day, with lights being turned off between 10:00 PM and 8:00 AM. The quality parameters of water [pH 6.5-7.5, NH_4^+ (< 1 mg/L), nitrite (< 1 mg/L), nitrate (< 20 ppm), and dissolved O_2 (8 ppm)] in each tank were monitored daily and remained within acceptable levels. Before starting the experiments, the fish were acclimated to the experimental condition and fed a commercial diet (AquaMax® Grower 400, Purina, MO) for 4 weeks. Fish with a similar body weight (the mean body weight of 16.6 g/fish) were assigned randomly into one of five treatment groups, with 3 tanks per group. Fish were hand-fed their respective diets twice daily at 09:00 and 16:00. Daily feed consumption of fish per tank (g/fish) was constant for all dietary groups. Total fish weight in each tank was recorded every two weeks to minimize handling stress. Fish were weighed after a 24-h

period of food deprivation. Almost 100% water in tank was replaced daily. The feeding trial lasted for 56 days.

Sample collection

At the beginning of the trial, 30 fish were randomly selected for euthanasia with 140 ppm MS-222 (neutralized by an appropriate amount of $NaHCO_3$), and their bodies were stored at – 80 ^oC for subsequent analysis of whole-body composition. At the termination of the feeding trial, all fish were starved for 24 h before weighing and sample collection. Then, total weights were recorded for each tank, and four fish per tank were randomly selected for the analysis of whole-body composition. Blood was collected with a hypodermic syringe from the caudal vein of conscious fish at 4 h after feeding. Blood samples were centrifuged at 8,000 *g* and 4 ^oC for 2 min. The supernatant (serum) was stored at −80 ^oC until analysis. After blood collection, all fish were euthanized, with three fish being randomly selected from each tank to obtain viscera organs [including the stomach and intestine (without luminal contents), as well as liver, pancreas, and spleen)], peritoneal adipose tissue, and skeletal muscle. For some of the anesthetized fish, the examination of their eyes was performed under darkened conditions using the LEICA ZOOM 2000 stereomicroscope with 4 or 10 magnification after fish were anaesthetizd with 140 ppm MS-222 (tricaine methane sulphonate). In this study, 6 fish with black skin syndrome were collected from different treatments to measure their body composition and morphometrical parameters. All tissue samples were frozen in liquid nitrogen and stored at -80 ^oC for subsequent chemical analyses.

Chemical analyses

AAs in serum was measured using HPLC, as described by Wu and Meininger (2008). Briefly, serum (0.1 mL) was deproteinized with 0.1 mL of 1.5 M $HClO_4$, followed by the addition of 0.05 mL of 2 M K_2CO_3. The mixture was centrifuged at 10 000g for 1 min, and the supernatant fluid was analyzed by HPLC (Waters, Milford, MA, USA). AAs in diets were analyzed after acid hydrolysis as described by Li et al. (2011). Crude protein (N × 6.25) was determined by the combustion method in Servi-Tech laboratories (Amarillo, TX, USA). Calcium and phosphorus were determined by optical spectrometry in Servi-Tech laboratories (Amarillo, TX, USA). Moisture was determined by drying at 105 ^{o}C in an oven to a constant weight. Lipids were extracted from the samples with chloroform/methanol (2:1 v/v) according to the method of Folch et al. (1957).

Histological analysis of tissues

Fresh tissues (~500 mg) from healthy fish and black-skin fish were fixed with 4% paraformaldehyde buffered at pH 7.2 for 24 h. The specimens were rinsed, dehydrated, and embedded in paraffin by Veterinary Medicine & Biomedical Sciences Histology Laboratory of Texas A&M University. Samples were cut into 6 μm transverse sections with a rotary microtome. The sections of the intestine were stained with periodic acid-Schiff (PAS) stain method, and sections of the liver and eyes were stained with hemotoxylin and eosin (H & E) method. The images were evaluated by using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axiocam HR digital camera. For the intestine, the radius of the organ, height of the villus and mucosa, width of the mucosa, submucosal thickness, and muscularis thickness were calculated by using the Axiovision 4.3 software.

Statistical analysis

Results are expressed as means ± SEM. All data were analyzed by using one-way ANOVA and the Student Newman Keuls multiple comparison test. They were tested for homogeneity (the Levene's test) and normal distribution (the Kolmogorov–Smirnov test) before analysis. When the variances of data were not homogenous, log transformations were performed before ANOVA. The Kaplan-Meier survival analysis was used to compare the cumulative survival rates of fish fed different diets. All analyses were performed by using the SPSS package (version 19.0, SPSS Inc, Chicago, IL, USA). Probability values ≤ 0.05 were taken to indicate statistical significance.

Results

Growth performance and feed utilization of LMB

Data on the growth rates of LMB are shown in Table VII-3. The body weights or weight gains of fish did not differ ($P > 0.05$) among the dietary treatments on day 14 of the trial. On day 28, the body weights or weight gains of fish fed the low fishmeal diets supplemented with either methionine or methionine plus taurine were increased ($P < 0.05$) in comparison with fish fed the FM diet or the unsupplemented low fishmeal diet. On day 56, the body weight or weight gains of fish were decreased ($P < 0.05$) in the unsupplemented low fishmeal group, compared with the whole fishmeal group. On all days of the experiment, taurine supplementation alone had no effect ($P > 0.05$) on the growth performance of fish. Importantly, at the end of the study, supplementation with either methionine or methionine plus taurine to the low fishmeal diet enhanced ($P < 0.05$) the body weight and weight gains of fish, compared with the FM group and the low fishmeal groups without methionine supplementation.

Feed utilization or the retention of dietary nitrogen and lipids in the body did not differ ($P > 0.05$) between fish fed the whole fishmeal diet and the unsuplemented low fishmeal diet (Table VII-4). Compared with the unsupplemented low fishmeal-based diets, dietary supplementation with either methionine or methionine plus taurine improved ($P < 0.05$) the feed intake (FI), protein efficiency ratio (PER) and feed conversion ratio (FCR) of LMB (Table VII-4), as well as the retention of dietary nitrogen and lipids in the body (Table VII-4). However, supplementation with taurine alone to the low fishmeal diet did not affect ($P > 0.05$) any of these variables, compared with the unsuplemented low fishmeal group. Either feed utilization or the retention of dietary nutrients in the body did not differ between fish fed the low fishmeal-based diets supplemented with methionine and methionine plus taurine.

Body composition and morphometrical parameters

Data on the body composition and morphometrical parameters are summarized in Table VII-5. At the end of the 56-day trial, the content of water, protein, and lipids in the body of largemouth bass was 71.0-71.5%, 17.5-18.3%, and 6.11-7.76%, respectively. Except for lipids, the composition of the whole body did not differ ($P > 0.05$) among the different groups of fish ($P > 0.05$). The lipid content of fish in the whole fishmeal group and in fish with black skin was lower ($P < 0.05$) than that in the other groups of fish. The viscerosomatic index (HSI) values of fish fed the low fishmeal diets with taurine supplementation or without any supplementation were lower ($P < 0.05$) than those in fish fed the whole fishmeal diet, and were improved ($P < 0.05$) in response to dietary supplementation with either methionine or methionine plus taurine. Fish with the black skin syndrome had a lower value of HSI than the healthy fish of all groups ($P < 0.05$). No

difference was detected ($P > 0.05$) in VSI or intraperitoneal fat ration (IPFR) among the different groups of fish.

Concentrations of AAs in serum

Concentrations of AAs in serum at 4 h after feeding are presented in Table VII-6. The concentrations of aspartate, glutamate, serine, arginine, lysine, and β-alanine in serum did not differ ($P > 0.05$) among the five groups of LMB, but those of all other measured AAs were affected ($P < 0.05$) by different treatments. Specifically, histidine concentrations in the serum of fish fed low-fishmeal diets with taurine or methionine supplementation or without any supplementation were higher ($P < 0.05$) than those in fish fed the whole fishmeal diet ($P < 0.05$). The concentrations of taurine in serum were markedly decreased ($P < 0.05$) in fish fed the low fishmeal diet either without taurine supplementation or with methionine supplementation alone when compared with fish fed the whole fishmeal diet, but were elevated ($P < 0.05$) in response to dietary supplementation with taurine. The concentrations of methionine in serum were decreased ($P < 0.05$) in fish fed the low fishmeal diet either without methionine supplementation or with taurine supplementation alone when compared with fish fed the whole fishmeal diet, but were elevated ($P < 0.05$) in response to dietary supplementation with methionine.

Interestingly, compared with fish fed the whole fishmeal diet, fish fed the low fishmeal diets without methionine or taurine supplementation had lower ($P < 0.05$) concentrations of glycine, threonine and isoleucine but higher ($P < 0.05$) concentrations of tyrosine, phenylalanine, tryptophan and valine in serum. Compared with the unsupplemented low fishmeal group, dietary supplementation with taurine increased ($P < 0.05$) the concentrations of citrulline, asparagine, ornithine, and lysine in serum, whereas

dietary supplementation with methionine increased ($P < 0.05$) the concentrations of asparagine, glutamine, glycine, and ornithine.

Black skin syndrome in fish fed low fishmeal diets

Fish fed the unsupplemented low fishmeal diets had black skin syndrome after 30 days of the feeding (Fig VII-1). A few fish fed the whole fishmeal diet developed the symptoms between days 41 and 43 of the experiment. Fish fed the low fishmeal diets supplemented with taurine plus methionine did not exhibit this metabolic problem before day 47 of the trial. At the end of the feeding trial, the prevalence of black skin syndrome was 38% in fish fed the unsupplemented low fishmeal diet, 22% in fish fed the low fishmeal diet supplemented with taurine, 11% in fish fed the low fishmeal diet supplemented with methionine, 10% in fish fed the low fishmeal diet supplemented with methionine plus taurine, and 5% in fish fed the whole fishmeal diet. Results of the Kaplan-Meier survival analysis indicated statistical differences ($P < 0.05$) among these groups of fish except for the two groups of fish fed the methionine-supplemented low fishmeal diets.

The eyes from fish with black skin syndrome were examined for health status with the use of a slit-lamp biomicroscope. All of the fish with black skin syndrome had damages to their eyes (Fig VII-2). Histological sections of eyes from these fish showed retinal degeneration (Fig VII-2). The liver from fish without black skin syndrome had normal sinusoid organization with normally shaped hepatocytes. However, necrosis, atrophy and sinusoid disorganization were present in the livers of fish with black skin syndrome. Furthermore, abnormal structure, atrophy and enteritis occurred in fish with black skin syndrome. Specifically, widening of the lamina propria was observed in intestinal mucosal samples from fish with black skin syndrome (Fig VII-4), which was infiltrated by a mixed

population of inflammatory cells (lymphocytes and macrophages, as well as eosinophilic, and neutrophilic granular cells). Compared with the whole fishmeal group, the radius and villus height, as well as mucosal height and width of proximal and distal intestines were lower ($P < 0.05$) in fish with black skin syndrome and in healthy fish in the unsupplemented low fishmeal group (Table VII-7). The thicknesses of the submucosa and muscularis of the proximal intestine did not differ ($P > 0.05$) among these groups of fish. In contrast, the thicknesses of the submucosa and muscularis of the distal intestine was lower ($P < 0.05$) in fish fed the unsupplemented low fishmeal diet than those in fish fed the whole fishmeal diet, but the values did not differ ($P > 0.05$) among fish fed the whole fishmeal diet, the low fishmeal diets supplemented with methionine or methionine plus taurine, and fish with black skin syndrome.

Discussion

Fishmeal contains high amounts and balanced proportions of proteinogenic AAs, as well as bioactive nutrients such as taurine, creatine, glutathione, and polyamines for animals, including fish (Li and Wu 2020). Thus, fishmeal has long been a staple protein feedstuff for fish, particularly carnivorous species (NRC 2011). However, it is not sustainable to feed fish with fish. The increasing global shortage and high price of fishmeal have prompted research to find its alternative sources (Hua et al. 2019). In this study, a mixture of non-fishmeal protein feedstuffs, which contained 45% poultry by-product meal, 30% soybean meal, 10% krill shrimp meal, and 15% blood meal, was used along with supplemental methionine or taurine to replace the dietary fishmeal content by 78%. This new nutritional strategy improved the growth and health of LMB, and is expected to play an important role in promoting the sustainability of the global aquaculture.

Selection of protein feedstuffs is crucial for successful replacement of fishmeal in diets for fish. A novel and significant result of the present study is that juvenile LMB fed the low protein diets supplemented with either methionine or methionine plus taurine had better growth performance and feed efficiency than fish fed the low fishmeal diet without methionine supplementation and even fish fed the whole fishmeal (Table VII-4). In contrast to methionine, supplementing taurine to the low fishmeal diets had no effect on the feed intake, body weight gains, or feed efficiency of LMB, but reduced the incidence of black skin syndrome. These findings indicate that the low fishmeal diet did not provide sufficient methionine for growth and health or sufficient taurine for health. Taurine is "conditionally essential" for some fish species (Li et al. 2009), but can be formed from methionine or cysteine in some fresh water fish species (EI-Sayed, 2014). However, whether this synthetic pathway is present in LMB is unknown. The fact that dietary supplementation with methionine to the low fishmeal diets did not affect the concentration of taurine in serum suggests that LMB does not synthesize taurine, as do carnivorous mammals (Wu 2020b). At present, little is known about the requirements of LMB for dietary taurine. Based on the content of taurine in the whole fishmeal diet and the unsupplemented low fishmeal diet (Table VII-2) as well as various health parameters, the dietary content of taurine for LMB should be > 0.31% and could be 0.6% (dry matter basis).

Growth performance is generally used as the primary criterion for assessing the efficacy of alternative protein feedstuffs to replace fishmeal in aquafeeds (Ren et al. 2018). However, the health status of fish fed a fishmeal replacement diet has often been ignored by researchers. In our previous work, we found that black skin syndrome occurred in LMB fed low fishmeal diets after about 4 weeks of feeding in our previous study (Chapter VI).

This metabolic problem was also observed in the present study, confirming that its outbreak started at day 30 in in fish fed an unsupplemented low fishmeal diet. Morphological color changes in skin have been described in teleost fish, amphibians, and reptiles due to alterations in the environment, diet, and season (Merchant et al. 2018; Sugimoto 2002; Whiteley et al. 2009). Interestingly, there is a report that the skin color of LMB can change after being housed in black tanks for few days (Moyer and Wilbur 1975). This color change results from ocular stimulation by light and is a rapid and reversible process (Merchant et al. 2018). However, LMB fed an unsupplemented low fishmeal diet exhibited black skin syndrome during a prolonged period of time (about 1 month), with the dark skin being persistent and independent on the background of water tanks. As a result, we suspect that the black skin syndrome in LMB is primarily due to eye damage that precludes fish from seeing light. In addition, a deficiency of methionine can impair the synthesis of glutathione in cells of the skin, leading to increased oxidative stress and the darkening of the tissue. As shown in Fig VII-5, fish with black skin syndrome had different degrees of retinal degeneration. Another eye disorder, cataract, has been diagnosed in many fish species, and can be induced by a variety of factors, including nutrition, environment, chemicals, or infections (Breck et al. 2003; Jonassen et al. 2017; Imsland et al. 2018; Remø et al. 2014; Trösße 2010). In all of those studies, cataract was detected with the use of slit-lamp biomicroscopy. Based on the pictures from the cataract cases (Trösße 2010), both diseases have similar symptoms under lamp biomicroscope inspection. In the present study, retinal damage was clearly evident in histological sections. It is necessary to diagnose retinal disorders by examinations by both histology and slit-lamp biomicroscopy.

The intestine and liver play important roles in digestion and absorption processes, and maintenance of these organs requires adequate intakes of dietary nutrients (Wu 2018). Another observation from the current work is that fish fed an unsupplemented low fishmeal diet developed intestinal and hepatic disorders. Histological analysis of the digestive system is considered a good tool with which to assess the nutritional status of fish (Rašković et al. 2011; Torrecillas et al. 2017). We found that most of the fish with black skin syndrome had non-infectious enteritis in the proximal and distal intestines. This intestinal disorder is consistent with its symptoms described by Baeverfjord and Krogdahl (1996) as the shortening of intestinal villi, the loss of supranuclear vacuolization of the enterocytes, the widening of lamina propria, and the infiltration of inflammatory cells in the lamina propria. This problem in Atlantic salmon can be induced by soyasaponins alone or by soyasaponins in combination with other factors, e.g., antigenic soybean proteins or changes in the intestinal microflora (Knudsen et al. 2007). Results of our study suggest that a high level of soybean meal as well as low levels of methionine and taurine in the low fishmeal diets may induce enteritis in LMB. In addition to the gut, liver damage, including necrosis, atrophy and sinusoid disorganization, was also observed in nearly all of the fish with black skin syndrome. This is consistent with the report of Rašković et al. (2011) that the most common changes in diet-induced abnormalities in the liver of fish are: hepatocyte vacuolization, fat and glycogen accumulation in the liver, changes in hepatic metabolic activity and parenchyma structure, and necrosis (Rašković et al. 2011). Notably, previous studies have shown that liver damage can be induced by many dietary factors, such as a deficiency of AAs (Espe et al. 2010), mycotoxins (Matejova et al. 2017; Santos et al. 2010), antinutrients in plant-source feedstuffs (such as soybean meal) (Evans et al. 2005),

imbalanced micronutrients (Tuan et al. 2002), and the peroxidation of polyunsaturated fatty acids (Du et al. 2008). Future studies are warranted to study oxidative stress in the digestive organs of fish fed low fishmeal diets.

An exciting finding from this study is that the prevalence of black skin syndrome in fish fed low fishmeal diets was reduced from 38% to 10% with methionine supplementation regardless of taurine provision (Figure VII-1). However, the rate of occurrence of black skin syndrome in fish fed methionine-supplemented low fishmeal diets was still higher than that (5%) in fish fed the whole fishmeal diet despite similar intake of dietary methionine (Table VII-2) and similar serum concentration of methionine (Table VII-6). Thus, factors other than methionine may also contribute to black skin syndrome in fish. In this regard, it is noteworthy that fish fed the low fishmeal diets had all elevated serum concentrations of tyrosine and tryptophan than fish fed the whole fishmeal diet (Table VII-6), despite similar dietary intake of tyrosine or tryptophan (Table VII-2). In animals, high concentrations of tryptophan and tyrosine in serum are often associated with hepatic dysfunction (Ninomiya et al. 1999; Laviano et al., 1997; Salerno et al. 1984). Interestingly, oral administration of high doses of tyrosine may cause retinal damage in rabbits (Weber et al. 1986). Likewise, in humans, hypertyrosinemia results in pathological manifestations the liver (Russo et al. 2001). It is possible that hepatic dysfunction in LMB may reduce the ability of their liver to degrade metabolites of black color (e.g., melanin and homogentisic acid from phenylalanine and tyrosine), resulting in their accumulation in tissues such as the skin. Furthermore, melatonin (a metabolite of tryptophan) can interfere with the metabolism and function of melanocytes of the skin (Kim et al. 2015), and so may also play a role in the onset of black skin syndrome in LMB. Further research is needed to

define a role for tyrosine and tryptophan in the pathogenesis of black skin syndrome in fish, including LMB.

Results of this study indicated that low-fishmeal (14.5%, dry matter basis) diets containing a mixture of 53.5% protein feedstuffs (45% poultry byproduct meal, 30% soybean meal, 15% blood meal, and 10% krill shrimp meal) did not provide adequate methionine for LMB; caused disorders in the intestine, liver and eyes, as well as black skin syndrome; and reduced the growth performance and feed efficiency of LMB. Regardless of taurine supplementation, adding 0.5% methionine to the low fishmeal diets improved the growth performance, feed efficiency, and health of LMB. In addition, our findings suggest that LMB may be incapable of synthesizing taurine from methionine, and a major metabolic function of dietary methionine may be related to anti-oxidative reactions to protect organs (e.g., intestine, liver, eyes, and skin) from oxidative damage. Further studies are warranted to understand the pathogenesis of the black skin syndrome in LMB.

Table VII-1 Composition and proximate analyses of the experimental diets

Ingredient (DM) basis)	FM	MP	MP + Tau	MP + Met	MP+ Tau & Met
Fishmeal	65.3	14.5	14.5	14.5	14.5
Mix protein[1]	---	53.5	53.5	53.5	53.5
Starch	7.0	5.0	5.0	5.0	5.0
Fish oil	3.8	3.8	3.8	3.8	3.8
Vitamin premix[2]	1.0	1.0	1.0	1.0	1.0
Mineral premix[3]	2.0	2.0	2.0	2.0	2.0
Cellulose	19.8	19.1	18.6	18.6	18.1
Carboxymethyl cellulose	1.0	1.0	1.0	1.0	1.0
Choline	0.14	0.14	0.14	0.14	0.14
Methionine	---	---	---	0.5	0.5
Taurine	---	---	0.5	---	0.5
Analyzed composition					
Dry matter (as-fed basis)	94.8	94.2	94.4	95.0	94.7
Crude protein (g/100 g DM)	45.5	45.5	45.8	45.8	46.1
Crude Fat (g/100 g DM)	9.54	9.83	9.77	9.64	9.95
Phosphorus (g/100 g DM)	2.16	1.32	1.22	1.24	1.28
Calcium (g/100 g DM)	3.53	2.05	1.93	1.97	1.95

[1] Mixture (g/100 g of a mix of protein feedstuffs): poultry by-product meal, 45 g; soybean meal, 30 g; Krill shrimp meal, 10 g; blood meal, 15 g.

[2] Vitamin premix (g/kg): vitamin A, 2.31; vitamin D3, 2.02; vitamin E, 20.00; vitamin K3, 1.2; vitamin C, 30.00; vitamin B5, 10.87; inositol, 15.00; niacin, 14.00; vitamin B6, 3.04; vitamin B2, 3.00; vitamin B1, 3.26; biotin, 0.15; folic acid, 0.6; vitamin B12, 0.02; Cellulose, 894.53.

[3] Mineral premix (g/kg): NaCl, 181.94; $MgSO_4.7H_2O$, 293.33; $FeSO_4.7H_2O$, 11.11; $AlCl_3.6H_2O$, 0.33; KI, 0.33; $CuSO_4.5H_2O$, 1.11; $MnSO_4$, 2.33; $CoCl.6H_2O$, 0.43; $ZnSO_4.7H_2O$, 9.04; Na_2SeO_3, 0.33; cellulose, 500.00.

DM = dry matter

Table VII-2 Composition of AAs in experimental diets (% of dry matter)

Amino acid	FM	MP	MP+ Tau	MP+ Met	MP+ Tau & Met
Asp	2.39	2.53	2.50	2.52	2.51
Asn	1.61	1.72	1.72	1.71	1.73
Glu	3.68	3.32	3.30	6.33	3.31
Gln	2.49	2.43	2.41	2.42	2.43
Ser	1.68	1.69	1.70	1.68	1.69
His	0.95	1.17	1.19	1.18	1.19
Gly	2.31	2.47	2.46	2.48	2.46
Thr	1.62	1.60	1.61	1.60	1.61
Arg	3.01	2.73	2.74	2.72	2.73
Ala	2.60	2.38	2.36	2.37	2.37
Trp	0.46	0.48	0.48	0.47	0.47
Tyr	1.35	1.37	1.368	1.37	1.36
Met	1.26	0.87	0.86	1.37	1.36
Val	1.89	2.11	2.13	2.10	2.12
Phe	1.63	1.85	1.86	1.85	1.84
Ile	1.68	1.57	1.56	1.56	1.55
Leu	2.86	3.18	3.17	3.16	3.17
Lys	3.03	2.76	2.74	2.75	2.76
Pro	2.30	2.49	2.51	2.50	2.48
Hyp	0.95	0.75	0.74	0.75	0.75
Cys	0.39	0.58	0.58	0.59	0.58
Tau	0.58	0.32	0.82	0.31	0.82
True protein	40.7	40.4	40.4	40.4	40.4

[1]Values are means for 3 samples of each feed analyzed. The content of proteinogenic amino acids in the diet was analyzed by HPLC after samples were hydrolyzed in 6 M HCl at 110 ^{o}C for 24 h (Li and Wu 2020), and calculated on the basis of the molecular weights of amino acids residues (i.e., the molecular weight of an intact amino acid – 18). Asn, Asp, Gln and Gln were analyzed using an enzymatic method (Li and Wu 2020). The content of taurine in diets (0.25 g) was determined by HPLC (Li and Wu 2020) after homogenization in 2 ml of 1.5 M $HClO_4$ and, then calculated on the basis of its intact molecular weight.

Table VII-3 Growth performance of largemouth bass fed fishmeal replacement diets[1]

Diet	Body weight (BW, g/fish)				Weight gain over the initial BW (%)		
	Initial	Day 14	Day 28	Day 56	Day 14	Day 28	Day 56
Whole fishmeal	16.6	28.4	36.6^{c}	55.5^{b}	71.6	120.7^{b}	234.9^{b}
MP	16.7	28.1	37.5^{bc}	51.4^{c}	68.0	124.2^{b}	207.1^{c}
MP + Tau	16.6	28.2	39.0^{ab}	53.3^{bc}	69.7	134.2^{a}	220.4^{bc}
MP + Met	16.7	29.1	40.1^{a}	63.7^{a}	74.8	140.7^{a}	282.5^{a}
MP + Tau & Met	16.6	28.8	39.9^{a}	62.2^{a}	73.8	140.3^{a}	274.9^{a}
SEM		0.19	0.42	1.37	1.15	2.50	8.41
P value		0.601	0.003	<0.001	0.340	0.003	<0.001

[1] Values are means with pooled SEM for 3 tanks of fish per treatment.
[a-c]: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).
MP, low fishmeal diet with a mix of protein sources

Table VII-4 Feed utilization and the retention (or productive value, %) of dietary nutrients in largemouth bass fed fishmeal replacement diets for 8 weeks[1]

Diet	Feed utilization			Retention of dietary nutrient in the body (%)			
	FI (g/fish)	FCR (g/g)	PER (g/g)	Nitrogen	Lipids	Phosphorus	Calcium
Whole fishmeal	43.7^{ab}	1.20^{ab}	1.82^{ab}	34.3^{b}	48.6^{b}	43.2^{b}	42.4^{b}
MP	40.5^{b}	1.27^{a}	1.68^{b}	33.4^{b}	45.5^{b}	56.5^{a}	55.0^{a}
MP + Tau	43.2^{ab}	1.27^{a}	1.68^{b}	32.5^{b}	49.5^{b}	61.0^{a}	56.3^{a}
MP + Met	47.9^{a}	1.08^{b}	1.97^{a}	36.3^{a}	62.8^{a}	64.7^{a}	59.4^{a}
MP + Tau & Met	47.6^{a}	1.11^{b}	1.89^{a}	36.2^{a}	61.4^{a}	62.5^{a}	60.2^{a}
SEM	0.85	0.03	0.03	0.50	2.20	2.39	1.80
P value	0.004	0.010	<0.001	0.158	0.007	0.003	0.006

[1] Values are means with pooled SEM for 3 tanks of fish per treatment.
$^{a-b}$: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).
MP, low fishmeal diet with a mix of protein sources

Table VII-5 Organ weight and body composition (% of wet body weight) in largemouth bass fed fishmeal replacement diets for 8 weeks[1]

Diet	Organ weight			Body composition				
	VSI	HSI	IPFR	Water	Protein	Lipids	Phosphorus	Calcium
Whole fishmeal	7.16	1.69[a]	1.85	71.5	17.8	6.11[c]	1.02	1.46
MP	6.56	1.31[b]	1.73	70.2	18.0	7.70[a]	0.92	1.39
MP + Tau	6.73	1.21[b]	2.09	70.5	18.2	7.18[b]	0.92	1.35
MP + Met	7.51	1.73[a]	2.16	70.8	17.7	7.51[a]	0.89	1.30
MP + Tau & Met	7.06	1.67[a]	1.96	70.0	18.3	7.76[a]	0.89	1.34
Fish with black skin syndrome	6.43	0.89[c]	1.79	71.1	17.5	6.31[c]	0.89	1.36
SEM	0.12	0.06	0.07	0.22	0.15	0.18	0.02	0.03
P value	0.143	<0.001	0.265	0.186	0.642	<0.001	0.089	0.093

[1] Values are means with pooled SEM for 3 tanks of fish per treatment.
[a-c]: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).
MP, low fishmeal diet with a mix of protein sources

Table VII-6 Concentrations (μM) of free AAs in the serum of juvenile largemouth bass at 4 hours after consuming fishmeal replacement diets[1]

Amino acid	Whole fishmeal diet	MP	MP + Tau	MP + Met	MP + Met & Tau	SEM	*P* value
Asp	36.6	38.3	36.5	34.4	33.9	2.22	0.906
Glu	57.8	57.3	61.2	56.3	60.3	2.65	0.942
Asn	146^{c}	121^{c}	332^{a}	220^{b}	336^{a}	17.6	<0.001
Ser	463	418	478	439	458	10.7	0.464
Gln	224^{b}	235^{b}	288^{a}	286^{a}	292^{a}	7.0	< 0.001
His	168^{b}	261^{a}	263^{a}	255^{a}	287^{a}	11.9	0.009
Gly	673^{a}	476^{c}	587^{b}	563^{b}	570^{b}	17.1	0.001
Thr	539^{a}	410^{b}	562^{a}	533^{a}	560^{a}	15.6	<0.001
Cit	102^{b}	101^{b}	135^{a}	101^{b}	139^{a}	4.34	<0.001
Arg	26.7	25.5	28.9	26.0	26.9	0.70	0.479
β-Ala	15.6	16.4	16.7	15.0	15.8	0.51	0.450
Tau	1733^{a}	1003^{b}	1824^{a}	1080^{b}	1883^{a}	77.2	< 0.001
Ala	1377^{b}	1402^{b}	1507^{b}	1543^{b}	1893^{a}	43.2	<0.001
Tyr	175^{b}	253^{a}	251^{a}	250^{a}	281^{a}	8.28	< 0.001
Trp	33.8^{b}	56.2^{a}	54.8^{a}	57.1^{a}	61.8^{a}	2.35	<0.001
Met	210^{b}	175^{c}	184^{c}	306^{a}	338^{a}	13.5.0	< 0.001
Val	761^{c}	895^{b}	936^{b}	873^{b}	1138^{a}	25.5	<0.001
Phe	224^{b}	302^{a}	287^{a}	306^{a}	337^{a}	8.6	0.005
Ile	360^{b}	312^{c}	271^{d}	309^{c}	420^{a}	10.7	<0.001
Leu	612^{b}	668^{b}	676^{b}	691^{b}	937^{a}	23.6	<0.001
Orn	149^{b}	145^{b}	209^{a}	190^{a}	208^{a}	7.1	<0.001
Lys	302	305	312	302	305	6.5	0.924

[1] Values are means with pooled SEM for 6 fish per treatment.

$^{a\text{-}c}$: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

MP, low fishmeal diet with a mix of protein sources

Table VII-7 Morphometrical measurements of proximal and distal intestines of largemouth bass fed fishmeal replacement diets[1]

Intestinal variable	FM	MP	MP + Tau	MP + Met	MP + Met & Tau	Fish with BSS	SEM	*P* value
Proximal intestine (PI)								
Radius of the organ (μm)	966.3^{a}	772.5^{c}	919.5^{ab}	1005.5^{a}	969.1^{a}	824.6^{bc}	19.0	<0.001
Height of the villus (μm)	570.9^{a}	446.5^{b}	581.1^{a}	570.5^{a}	589.1^{a}	467.6^{b}	11.2	0.001
Height of the mucosa (μm)	685.6^{a}	578.4^{b}	686.4^{a}	685.2^{a}	692.9^{a}	559.7^{b}	12.6	<0.001
Width of the mucosa (μm)	104.3^{a}	94.1^{b}	118.8^{a}	109.6^{a}	110.0^{a}	96.6^{b}	2.20	0.009
Submucosal thickness (μm)	36.2^{b}	42.1^{a}	32.9^{b}	33.5^{b}	33.1^{b}	34.0^{b}	0.90	0.383
Muscularis thickness (μm)	80.9	83.7	71.7	75.5	84.4	83.5	1.97	0.275
Distal intestine (DI)								
Radius of the organ (μm)	890.1^{a}	717.5^{b}	852.8^{a}	859.2^{a}	845.4^{a}	649.4^{b}	16.3	<0.001
Height of the villus (μm)	523.8^{a}	463.2^{b}	485.4^{b}	472.4^{b}	513.5^{a}	383.4^{c}	8.59	<0.001
Height of the mucosa (μm)	627.1^{a}	484.0^{c}	550.7^{b}	538.2^{b}	548.5^{b}	445.3^{c}	10.1	<0.001
Width of the mucosa (μm)	119.8^{a}	97.7^{b}	96.4^{b}	115.6^{a}	114.5^{a}	102.4^{b}	2.40	0.003
Submucosal thickness (μm)	31.2^{a}	19.2^{b}	28.1^{a}	29.4^{a}	27.8^{a}	26.4^{a}	0.96	0.004
Muscularis thickness (μm)	86.9^{a}	67.9^{b}	87.8^{a}	100.4^{a}	85.2^{a}	97.1^{a}	2.49	<0.001

[1]Values are means with pooled SEM for 3 tanks of fish per treatment.

a-c: Within a row, means not sharing the same superscript letter differ ($P < 0.05$).

BSS, black skin syndrome; FM, whole fishmeal diet; MP, low fishmeal diet with a mix of protein sources

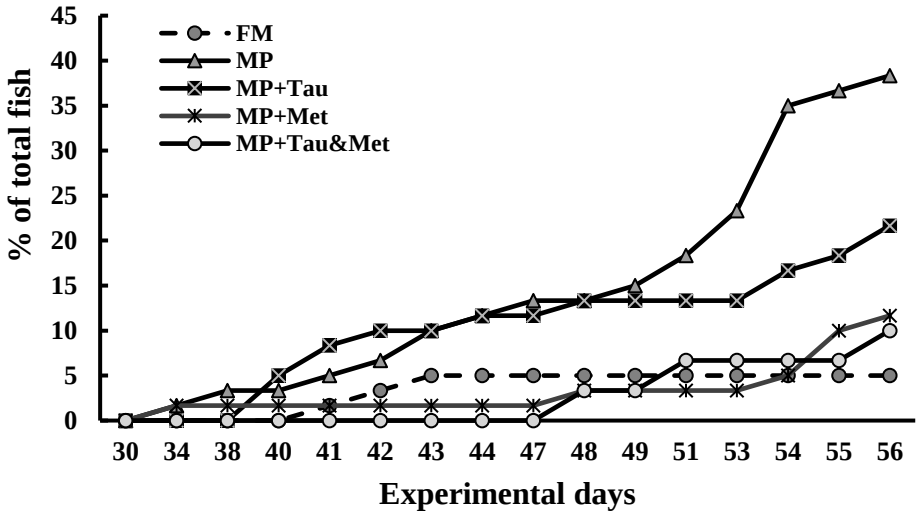


Figure VII-1 The accumulative rates of black skin syndrome of fish fed fishmeal replacment diets for 8 weeks At the end of the feeding trial, the prevalence of black skin syndrome was 38% in fish fed the unsupplemented low fishmeal diet, 22% in fish fed the low fishmeal diet supplemented with taurine, 11% in fish fed the low fishmeal diet supplemented with methionine, 10% in fish fed the low fishmeal diet supplemented with methionine plus taurine, and 5% in fish fed the whole fishmeal diet. Results of the Kaplan-Meier survival analysis indicated statistical differences ($P < 0.05$) among these groups of fish except for the two groups of fish fed the methionine-supplemented low fishmeal diets.

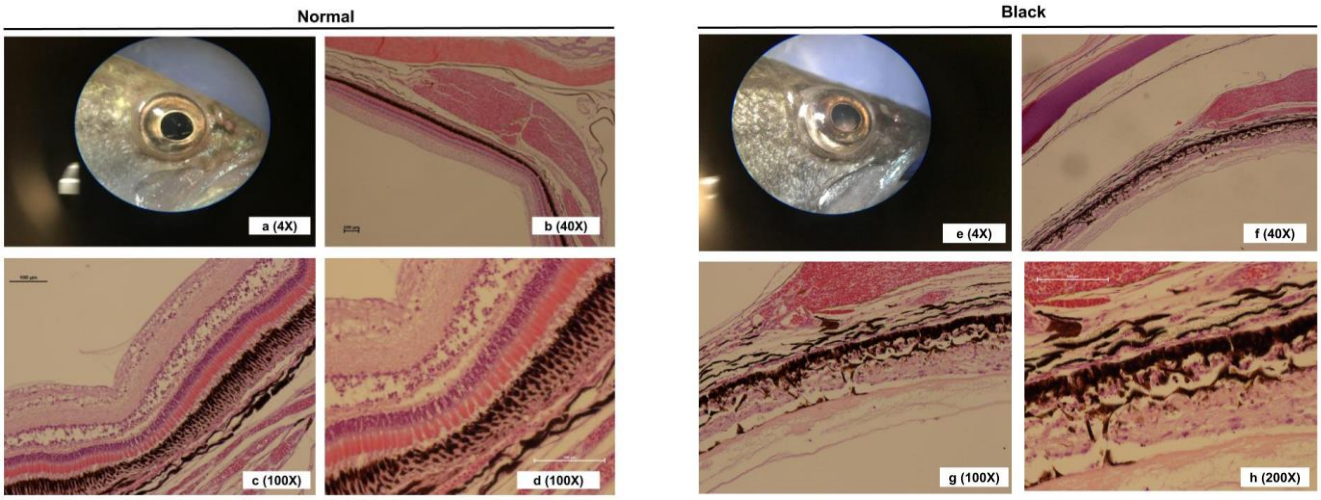


Figure VII-2 Retinal degeneration in fish with black skin syndrome. a, b, c, and d: Normal fish with the healthy retina; e, f, g, and h: Fish with black skin syndrome including retinal damage

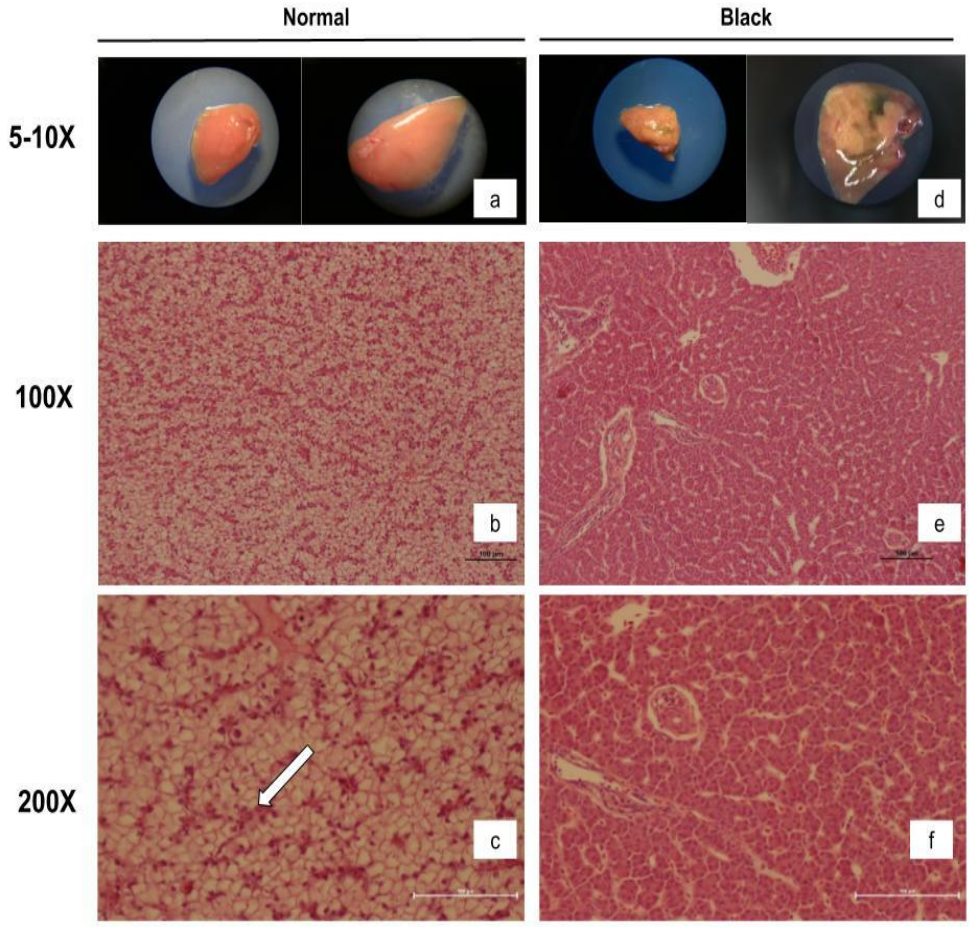


Figure VII-3 Damage to the liver in fish with black skin syndrome. a, b, c: Normal fish that had the healthy liver with normal sinusoid organization (white arrow) and well-shaped hepatocytes. d, e, f: Fish with black skin syndrome, including necrosis, atrophy and sinusoid disorganization of the liver

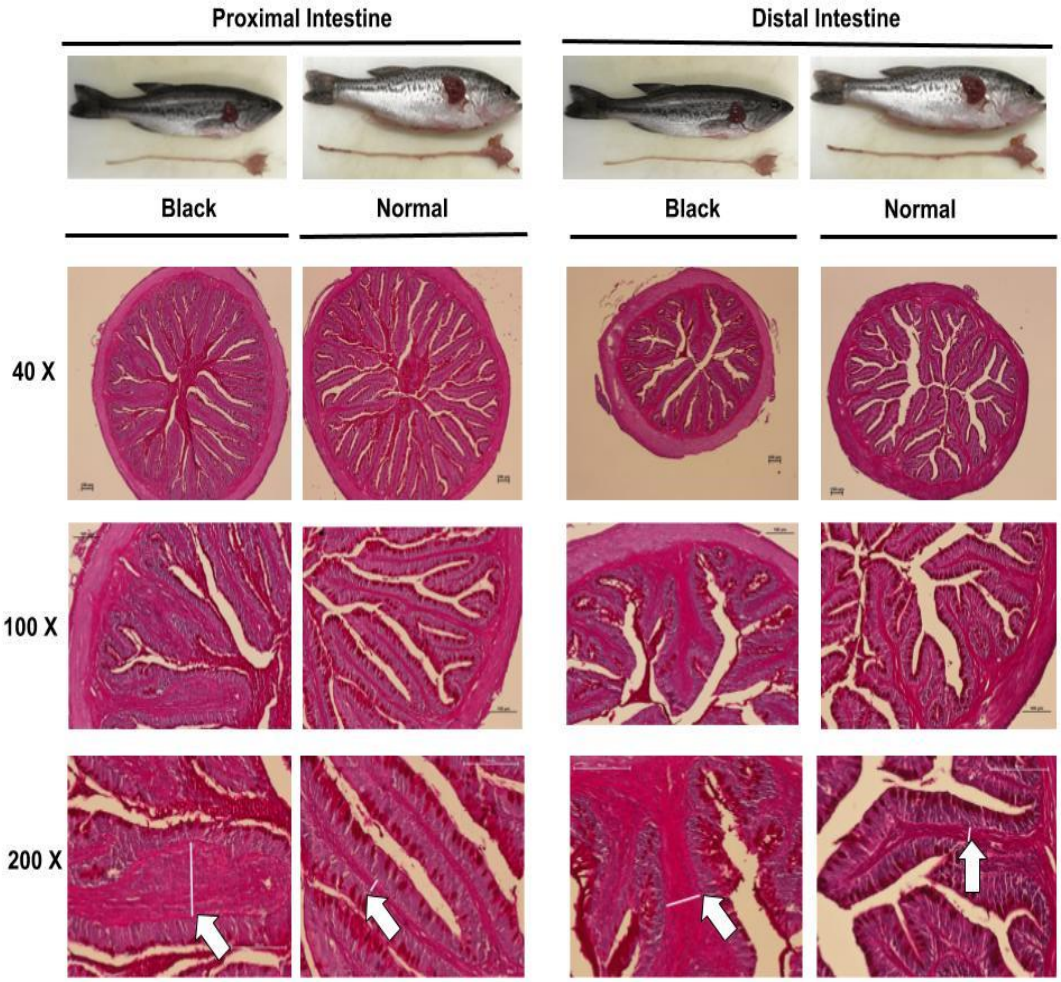


Figure VII-4 Intestinal imflammation in fish with black skin syndrome. White arrow: Compared with healthy fish, fish with black skin syndrome had wider lamina propria with more inflammatory cells, indicating inflammation in the lamina propria

CHAPTER VIII

CONCLUSIONS

Aquaculture is increasingly important for providing humans with high-quality animal protein to improve growth, development and health. More than 70% of the production cost is dependent on the supply of compound feeds. A public debate or concern over aquaculture is its environmental sustainability because many fish species have high requirements for dietary protein and fishmeal. Protein or amino acids, which are the major component of tissue growth, are generally the most expensive nutrients in animal production and, therefore, are crucial for aquatic feed development. Understanding the metabolism and functions of protein and amino acids is fundamental for manufacturing environment-friendly aquafeeds and reducing dietary protein level and feed costs in fish production. The major findings from this dissertation research are to: provide new knowledge about the oxidation of amino acids, glucose, and fatty acids as metabolic fuels in tissues of largemouth bass (LMB); reveal metabolic disorders (hepatic glycogenosis and black skin syndrome) in fish fed with high starch or low fishmeal diets; and develop nutritional methods to replace fishmeal in the diets for LMB.

The first important finding from our work is that amino acids are the main substrates for ATP production in LMB. We founded that LMB had a higher ability to utilize glutamate and glutamine as energy substrates than glucose and fatty acids. Glutamine plus glutamate plus aspartate generated 60-70% of ATP in LMB tissues. Moreover, the rates of amino acid oxidation in these tissues could be regulated by dietary protein intake in a

tissue-specific manner to meet their metabolic needs. Based on these results, we surmise that fish have particularly high requirements for dietary protein to primarily satisfy their needs for glutamate, glutamine and aspartate. Therefore, we propose that addition of low-cost, feedstuff sources of glutamate, glutamine, and aspartate to low protein diets may help to reduce the use of fishmeal in the diets for LMB. This mechanism-based innovative practice is expected to sustain aquaculture in the face of diminishing resources.

Feeding LMB with low-protein diets impairs their growth. In those diets, starch content is usually high. The present study identified that this fish cannot tolerate a high dietary starch level that is typically associated low-protein intake. We found that hepatosis occurred in LMB fed diets containing ≥ 10% starch because of hepatic glycogenosis or glycogenic hepatopathy that resulted from prolonged elevations of blood glucose concentrations to promote glycogen synthesis in the liver. Our findings indicated that the liver, proximal intestine, kidney, and skeletal muscle had a limited ability to oxidize glucose and palmitate for ATP production, compared with glutamate, glutamine and aspartate, due to low activities of carnitine palmitoyltransferase-I, hexokinase and pyruvate dehydrogenase. The liver of LMB had a high capacity for synthesizing glycogen from glucose and a much lower capacity for converting glucose into fatty acids. Thus, when abundant glucose is supplied to the liver, this nutrient is converted into glycogen and water retention. These findings further explain, in part, why LMB fed commercial diets containing high starch levels develop hepatic disorders and rapidly accumulate excessive fats in the body. Such a metabolic disorder may also occur in other fish species, which impairs their growth and causes their deaths. Accordingly, we recommended

dietary crude protein, lipids and starch levels to be 45%, 10% and < 10%, respectively, for juvenile LMB. Our results may have important implications for understanding metabolic syndrome in humans who are susceptible to high dietary starch intake.

Knowledge of nutrient metabolism in LMB led to two studies for fishmeal replacement in the diets for the fish. Our findings indicated that an inclusion of 15% fishmeal protein in the diet (dry matter basis) was sufficient for LMB growth. However, we discovered that some of the fish that were fed diets containing ≤ 15% fishmeal protein had black skin syndrome, characterized by not only black spots in the skin but also damages in the eye, intestine and liver. The black skin syndrome may be primarily due to retinal disorders, which are related to the deficiency of methionine. Our findings suggested that adding 0.5% methionine to the low fishmeal diets improved the growth performance, feed efficiency, and health of LMB. The prevalence of black skin syndrome in fish fed low fishmeal diets was reduced from 38% to 10% with methionine supplementation. Another important finding from this work is that concentrations of tyrosine and tryptophan in serum were higher in fish fed diets with low fishmeal protein than those in the whole fishmeal-protein group. Excess tyrosine (hypertyrosinemia) and tryptophan (hypertryptophanemia) in serum are toxic to animals.

Collectively, findings from the present research not only advance basic knowledge of the nutrition, metabolism and functions of AAs in fish species, but also provide the much needed biochemical and nutritional bases for developing next generation of practical feeds for LMB to enhance the efficiency of their growth and sustain their global

production. Moreover, the outcome of the project also designs new aquafeeds to improve the growth performance of fish and the health of their gut, eyes and liver.

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APPENDIX A

Glycogen and Glucose Assay (UV Spectrophotometric Method)

Glycogen hydrolysis:

1. For each single sample, prepare 0.5 ml of 1.0 M HCl in a 2 ml Eppendorf tube. As the control to measure glucose, substitute 1.0 M HCl with 1.0 M NaOH.

2. Heat the tubes in boiling water for 3 to 5 min.

3. Centrifuge the tubes briefly, and then wipe the tubes and record the weights shown on an analytical balance.

4. Transfer 20 - 30 mg samples into the above tubes with hot HCl or NaOH.

5. Seal the tubes tightly and boil the samples in a water bath for 3 h. To achieve complete hydrolysis, mince the samples with scissors, and then shake the tubes vigorously every 30 min during the whole process.

6. Cool samples on bench to room temperature (RT, 25 ^{o}C). Neutralize the hydrolysis product with 0.5 ml of 1.0 M NaOH or HCl, accordingly.

7. Vortex the tubes vigorously and then centrifuge them at speed (*i.e.*, ~ 10,000 *x g*) for 5 min to remove the insoluble pellet.

Bring the sample to a final volume of 100 and 5 ml for liver and muscle, respectively.

Glucose determination:

Glucose concentration can be determined using the following method.

1. For each test tube, add 100 µl reaction solution (0.1M TEA, 2 mM ATP, 1.27 mM NADP-Na_2, 50 µg/ml glucose-6-phosphate dehydrogenase (G-6-PDH), 100 µl sample

solution or standard (0 - 300 nmol/ml) and 2 µl $MgCl_2$ (50 mM). Mix the solution thoroughly.

2. After 5 min, take 200 µl reaction solution for optical density (OD) reading (OD_1) at the wavelength of 340 nm.

3. Add 1 µl of hexokinase (2 mg/ml) to each test tube. Mix the solution gently. After 5 min, take 200 µl solution for OD reading (OD_2) at 340 nm.

Glycogen content is determined as:

1. Absorbance (Abs) = $OD_2 – OD_1$
2. Abs for sample/Abs for standard x Concentration (standard) x Total volume / Weight (sample, g)
3. Unit = mg of glycogen/g of wet tissue weight.

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.

3.

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.

3.

Determination of Glutamate-Oxaloacetate Transaminase (GOT) Activity

A. Chemicals

4. 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.
5. 2.4 mM NADH: Dissolve 35 mg NADH (disodium salt) in 20 ml of 80 mM sodium phosphate buffer (pH 7.6).
6. 210 mM α-Ketoglutarate: Dissolve 238 mg α-ketoglutarate (disodium salt) in 5 ml of 80 mM sodium phosphate buffer (pH 7.6).
7. 38 mM Aspartate: Dissolve in 506 mg aspartic acid in 100 ml of 80 mM sodium phosphate buffer (pH 7.6).
8. Malate dehydrogenase: Use stock solution undiluted.

B. Assay procedures:

4. 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
5. 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 NADP: 5 mg NADP (mw: 551.14 (anhydrous basis)) in 1 ml Tris·$MgCl_2$ buffer. (Prepare fresh)
   5. Glucose-6-phosphate dehydrogenase (G6PD) 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
NADP solution	1.6 ml
G6PD 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 monopatassium salt) 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℃. 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℃ 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℃.
   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℃.
   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 the tube 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.

Preparation of KHB medium

A. Mixture media

1. 1 μM Insulin (MW: 5808): Dissolve 1 mg bovine insulin in 1 ml of 167 mM $NaHCO_3$. Vortex.

2. 2 mM palmitate: Add 20.6 palmitate in 50 ml KHB is solubilized in 0.43% BSA. The conjugation of palmitate with BSA by stirring takes ~ 4 h at 37 ºC.

3. KHB media (2 mM palmitate, 5 mM Glucose and 0.1 nM insulin): Dissolve 51.2 mg palmitate acid, 90 mg D-glucose and 10 μl of 1 μM Insulin into 100 ml of KHB (pH 7.4, Gas the solution for 10 min with 95% O_2/5% CO_2).

4. Add other substrates for as follows:

(a) 2 mM L-leucine (MW 131.2): Add 13 mg L-Leucine to the 50 ml media;

(b) 2 mM L-glutamine (MW 146.13): 15 mg L-Glutamine to the 50 ml media;

(c) 2 mM L-glutamate (MW 147.13): 15 mg L-Glutamate to the 50 ml media;

(d) 2 mM L-aspartate (MW 133.11): 14 mg L-aspartate to the 50 ml media;

(e) 2 mM L-alanine (MW 89.09): 9 mg L-alanine to the 50 ml media.

B. Single medium: Prepare the KHB buffer with each substrate separately.

Transport of nutrients in LMB tissues

A. ^{3}H-Inulin stock solution: Add 20 μl 3H-inulin to 0.5 ml KHB medium.

B. Prepare incubation medium with a tracer

1. 2 mM [U-^{14}C]Leucine (Leu): Mix 7 μl of [U-^{14}C]leucine, 50 μl of ^{3}H-inulin solution, and 20 ml of mixture-substrate medium. Count 100 μl of the solution for ^{14}C.

2. 2 mM [U-^{14}C]Palmitate acid (PA): Mix 7 μl of [U-^{14}C]palmitate, 50 μl of ^{3}H-inulin solution, and 20 ml of mixture-substrate medium. Count 100 μl of the solution for ^{14}C.

3. 5 mM [U-^{14}C]Glucose (Gluc): Mix 1 μl of [U-^{14}C]glucose, 50 μl of ^{3}H-inulin solution, and 20 ml of mixture-substrate medium. Count 100 μl for ^{14}C.

4. 2 mM [U-^{14}C]Glutamine (Gln): Mix 7 µl of [U-^{14}C]glutamine, 50 µl of ^{3}H-inulin solution, and 20 ml of mixture-substrate medium. Count 100 µl of the solution for ^{14}C.

5. 2 mM [U-^{14}C]Glutamate (Gln): Mix 7 µl [U-^{14}C]glutamate, 50 µl of ^{3}H-inulin solution, and 20 ml of mixture-substrate medium. Count 100 µl of the solution for ^{14}C.

6. 2 mM [U-^{14}C]Aspartate (Asp): Mix 7 µl of [U-^{14}C]aspartate, 50 µl of ^{3}H-inulin solution, and 20 ml of mixture-substrate medium. Count 100 µl of the solution for ^{14}C.

7. 2 mM [U-^{14}C]Alanine (Ala): Mix 7 µl of [U-^{14}C]alanine, 50 µl of ^{3}H-inulin solution, and 20 ml of mixture-substrate medium. Count 100 µl of the solution for ^{14}C.

C. Tissue incubation

1. Cut muscle, liver, kidney and intestine into small pieces.

2. Add a tissue (20-50 mg) into a tube with 1 ml (Make sure to weigh sample for each tube.)

3. Incubate the tubes at 26°C for 5 min.

4. At the end of 5 min incubation, wash the tissue rapidly 3 times with cold PBS (in 3 different bottles).

5. Add 0.5 ml of 1 M NaOH to tissue and place the tube in a 50 ^{o}C waterbath until the tissue is dissolved.

6. Transfer 0.1 ml of the solution to a 5-ml liquid scintillation vial. Add 5 ml of scintillation cocktail (for Liquid Sample) to each vial for ^{14}C and ^{3}H dual counting.

Determination of oxidation of energy substrates

A. Tissue incubation

1. Cut tissues into small pieces.

2. Add a tissue (20-50 mg) into a tube with 1 ml medium. (Make sure to weigh sample for each tube.)

3. Gas each tube for 20 sec with 95% O_2/5% CO_2. Incubate the tubes at 26°C for 2 h.

B. CO_2 collection.

1. At the end of 2-h incubation, inject 0.2 ml of Soluene-350 into the center-wall cup within the tube through the stopper. Then inject 0.2 ml of 1.5 M $HClO_4$ into the incubation medium through the stopper.

2. Incubate the tubes at 26 °C for 1 h for collecting CO_2.

3. At the end of 1-h incubation, transfer the cup in the center-well of each tube to a 5-ml liquid scintillation vial. Add 5 ml of ORGANIC cocktail to each vial for $^{14}CO_2$ counting.

4. Save the incubation medium plus tissue for subsequent analysis.

5. Calculation: CO_2 production = DPM of collected $^{14}CO_2$/Specific activity of substrate in medium;

Specific activity of a tracer (DPM/nmol) = DPM of tracer in medium/nmol of tracee

When the number of carbons in each molecule is considered, the specific activity of a tracer is expressed as DPM/nmol C.

Determination of glycogen synthesis

A. Tissue incubation: The processes of tissue incubation are the same as above steps in the substrate oxidation experiment involving a ^{14}C-labeled substrate.

B. Glycogen extraction

1. At the end of the incubation, centrifuge the tubes with incubation medium (1 ml) for 3 min at 3500 rpm and obtain the pellet (tissue). Wash the pellet with ice-cold PBS for 5 times.

2. Add 0.3 ml of 30% KOH (5.3M) to the tissue/tube and boil the tube in a water bath for 5 min.

3. Add 0.1 ml of 2% Na_2SO_4 and 0.9 ml of 100% alcohol to the tube. The tubes stand at 25 ^oC overnight.

4. The pellet (glycogen-sodium sulphate) is obtained by centrifuging at 3500 rpm for 10 min. The supernatant fluid is saved for extraction of glycogen associated with protein (See Step #6 below).

5. For the pellet glycogen fraction: The tube walls and pellet are washed with 2 ml of 65% alcohol two times. When the alcohol residue is evaporated in a water bath at 70 ℃, the precipitate is dissolved in 0.1 ml of 1N H_2SO_4. The solution is transferred to a glass counting vial with 5 ml of scintillation cocktail and counted by LSC;

6. For the supernatant glycogen fraction: The supernatant fluid is neutralized with 6 N HCL (about 265 ul). Then, 1 ml of 1.5M $HClO_4$ is added to the supernatant fluid. The pellet is obtained by centrifuging for 10 min at 3500 rpm. The pellet is washed with 2 ml of 1.5 M $HClO_4$ three times and then dissolved in 0.3 ml of 30% KOH. The solution is neutralized with 6 N HCl (265 ul) and 0.1 ml of the solution is measured for ^{14}C radioactivity by a liquid scintillation counter.

7. Add 5 ml scintillation cocktail for counting ^{14}C by a liquid scintillation counter.

Determination of fat synthesis

A. Tissue incubation: The processes of tissue incubation ia same as steps in the oxidation experiment involving a ^{14}C-labeled substrate.

B. Lipid extraction

1. Extraction mixture: Isopropylalcohol:Heptane:1N H_2SO_4 (40:10:1).

2. Add 5 ml of extraction mixture to incubation medium and tissue, homogenates it for 2 min.

3. Vortex the tube for at least 1 min. Wait 5-10 min;

4. Add 3 ml Heptane and 3 ml H_2O to the tube.

5. Cap the tubes, and vortex them again.

6. Wait 5-10 mins until layers separate. Transfer the upper layer (heptane layer) that contains lipids to a scintillation vial.

7. Dry down the collected layer to less than 0.5 ml.

8. Add 5 ml of scintillation cocktail to the scintillation vial for counting ^{14}C by a liquid scintillation counter.

Determination of O_2 consumption using a 6-channel oxygen meter (Strathkelvin Model 929)

1. Gas KHB medium for 15 min with 95% O_2/5% CO_2 before use.

2. Turn on water bath and set up temperature at 26 ℃.

3. Cut tissues into small pieces.

4. Add a tissue (10-20 mg for liver, kidney and intestine; and 40 mg for skeletal muscle) into each chamber with 1 ml KHB medium.

5. All tissues were gently incubated with stirring at 26 ℃ for 30 min. The oxygen concentration was recorded over a 20-min period.

6. The rate of oxygen consumption by a tissue was generated by the software of the instrument based on the change of oxygen concentration in each chamber solution between 10 and 20 min when oxygen concentration in the medium linearly decreased.

Hematoxylin and eosin (H&E) staining protocol

Sectioning			
Keep block on mollifex	30~45 min		
Keep block on ice	~30 min		
Tissues are cut at around 6 µm			
Once cut, the tissue ribbons are carefully transferred to a water bath in 37 ℃. Here they are allowed to float on the surface, and can then be scooped up onto a slide placed under the water level.			
Dry slide overnight at 37 ℃			
Store the slides in 4 ℃ until use			
Warm up the slides at 55℃ for 15 min before staining			
Staining			
Solution	**Time (min)**	**Times**	**Comments**
Citrasolve	5	4	Agitate every 2 min
100% Ethanol	3	3	
95% Ethanol	1	3	
70% Ethanol	3	3	
Distilled Water	5	2	
Fresh filtered Harries-Hematoxylin solution	~ 10 sec (Intestine 7s, Liver 10s)	1	Check for intensity using a miscroscope; can hold the slide in water
Distilled water	1	~7	Until water with no color
Ammonia water (0.1%)	~ 5 sec	2	
Distilled Water	1	3	
70% Ethanol	1	2	
Esion Y	Second~10	1	Check for intensity using a microscope; can hold the slide in 95% ethanol
95% Ethanol	1	3	
100% Ethanol	1	2	
Citrasolve	5	4	Agitate every 2 min
Place a drop of permount on the slide using a glass rod, taking care to leave no bubbles.			
Angle the coverslip and let it fall gently onto the slide. Allow the permount to spread beneath the coverslip, covering all the tissue.			
Dry the slides overnight in the hood.			

Hematoxylin and PAS staining protocol

Sectioning			
Keep block on Mollifex	30~45 min		
Keep blocks on ice	~30 min		
tissues are cut at around 6-8 µm			
Once cut, the tissue ribbons are carefully transferred to a water bath in 37 ℃. Here they are allowed to float on the surface, and can then be scooped up onto a slide placed under the water level.			
Dry slide overnight at 37℃			
Store the slides at 4 ℃ until use			
Warm up the tubes at 55℃ for 15 min before staining			
Staining			
Solution	**Time (min)**	**Times**	**Comments**
Citrasolve	5	4	Agitate every 2 min
100% Ethanol	3	3	
95% Ethanol	1	3	
70% Ethanol	3	3	
Distilled Water	5	2	
0.1% Amylase solution	~20 sec	1	For removing glycogen only
1% Periodic acid	10	1	
Distilled Water	1	~5	
Schiff regent	15-20	1	Check for intensity using a microscope
Tap water	5-10	Running water	Check the red color intensity
Fresh Filtered Harries-Hematoxylin	~ 10 sec (Intestine, 7 sec; Liver 10 sec)	1	Check for intensity using a microscope; can hold the slide in water
Distilled water	1	~7	Until water clear
Ammonia water (0.1%)	~ 5 sec	2	
Tap Water	3	Running water	
70% Ethanol	1	2	
95% Ethanol	1	3	
100% Ethanol	1	2	
Citrasolve	5	4	Agitate every 2 min
Place a drop of permount on the slide using a glass rod, taking care to leave no bubbles.			
Angle the coverslip and let it fall gently onto the slide. Allow the permount to spread beneath the coverslip, covering all the tissue.			
Dry the slides overnight in the hood.			