THE ROLE OF BRANCHED-CHAIN AMINO ACIDS IN THE AQUACULTURE OF RED DRUM, *Sciaenops ocellatus* L. − DEFINING DIETARY REQUIREMENTS AND ELUCIDATING ANTAGONISTIC EFFECTS

A Dissertation

by

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ABSTRACT

Five separate feeding trials were conducted to investigate branched-chain amino acid (BCAA) nutrition of red drum (*Sciaenops ocellatus*). First, the minimum dietary requirements of the BCAAs (leucine [Leu], isoleucine [Ile] and valine [Val]) were determined for juvenile red drum. Three independent 49-d feeding trials were conducted. Experimental diets were prepared by supplementing a basal diet containing 37 g/kg crude protein (CP) from red drum muscle (RDM) and crystalline amino acids (AAs) with incremental levels of Leu (9.0, 13.0, 17.0, 21.0, 25.0 and 29.0 g/kg of dry diet), Ile (5.0, 8.0, 11.0, 14.0, 17.0 and 20.0 g/kg of dry diet) and Val (6.8, 8.0, 9.2, 10.4, 11.6, 12.8 and 14.0 g/kg of dry diet). Fish in triplicate 30-L glass aquaria were fed each experimental diet to apparent satiation twice daily, after which growth performance parameters were measured. Incremental levels of dietary Leu, Ile and Val significantly affected weight gain, feed efficiency and protein retention. Analyses of the weight gain data using a broken-line regression model estimated the minimum Leu, Ile and Val requirements for maximum weight gain of juvenile red drum to be 15.7 ± 1.7 g/kg (± 95% confidence interval), 11.1 ± 2.3 g/kg and 12.4 ± 0.6 g/kg of dry diet, respectively.

Secondly, the effects of high dietary levels of BCAAs on growth performance and AA utilization of juvenile red drum were determined. A semi-purified control diet (430 g/kg CP) was prepared by combining lyophilized RDM and crystalline AAs, while keeping the levels of Leu, Ile and Val at the minimum dietary requirement for red drum. Six experimental diets were prepared by supplementing the control diet with (1) an excess of Leu (62.0 g/kg of dry diet), (2) an excess of Ile (44.0 g/kg of dry diet), (3) an excess of Val (50.0 g/kg of dry diet), (4) an excess of Leu and Ile (62.0 and 44.0 g/kg), (5) an excess of Ile and Val (44.0 and 50.0 g/kg), and (6) an excess of Leu and Val (62.0 and 50.0 g/kg). Excess levels of Leu, Ile and Val corresponded to 200% of the amount found in a diet with 350 g/kg CP provided by RDM. Red drum juveniles were stocked in 38-L glass aquaria (12 fish/aquarium), and diets were fed to fish in triplicate aquaria at a rate approaching apparent satiation, twice daily, for 49 d. At the end of the feeding trial, growth performance parameters were calculated and the postprandial concentration of BCAAs in plasma was analyzed. Growth performance of the red drum was significantly affected by an excess of dietary Leu, alone or in combination (Leu+Ile and Leu+Val), confirming an antagonistic effect due to the imbalanced concentration of BCAAs in the diet. In addition, the postprandial concentration of plasma Leu, Ile and/or Val was significantly higher in fish fed an excess of Leu, Ile and/or Val, respectively. Nonetheless, postprandial levels of BCAAs in plasma did not indicate that an excess of Leu blocked the intestinal absorption or promoted the catabolism of Ile and/or Val in red drum, as has been reported in other species.

Ultimately, the results obtained from this dissertation will facilitate the production of aquaculture feeds composed of alternative protein ingredients while maintaining an ideal AA profile for optimal performance of red drum.

DEDICATION

Para Ana, mi inspiración.

“Dos almas no se encuentran por casualidad” – Jorge Luis Borges

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NOMENCLATURE

α-KG	α-Ketoglutarate
AA	Amino Acid
ANOVA	Analysis of Variance
BCAA	Branched-chain Amino Acid
BCKA	Branched-chain α-Keto Acid
BCKDH	Branched-chain α-Keto Acid Dehydrogenase Kinase
CI	Confidence Interval
FE	Feed Efficiency
HSI	Hepatosomatic Index
IAA	Indispensable Amino Acid
IGF	Insulin-like Growth Factor
Ile	Ile
IPF	Intraperitoneal Fat
Leu	Leucine
mTOR	Mammalian Target of Rapamycin
PRE	Protein Retention Efficiency
RDM	Red Drum Muscle
UPLC	Ultra-Performance Liquid Chromatography
Val	Valine

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

TABLE Page

CHAPTER I

INTRODUCTION, LITERATURE REVIEW AND RESEARCH GOALS

I.1 Aquaculture and Amino Acid Requirements

Nowadays, fish represent an important source of nutritious food and animal protein for most of the world's population. Accordingly, aquaculture at the moment is one of the fastest growing animal food-producing sectors, supplying the world with about 73.8 million tonnes of food fish, valued at US$160.2 billion in 2014 (FAO, 2016). In the last five decades, world fish food supply has kept pace with global population growth, and aquaculture has expanded from being almost negligible to fully comparable to capture fisheries in terms of providing seafood for feeding people in the world (FAO, 2016).

Given the state of the world's fisheries, where most of the fish stocks are fully exploited and, therefore, have no potential for increases in production, future demand for seafood and fisheries products can only be met by an expanded aquacultural production. Such production will likely become more intensive and increasingly depend on nutritious prepared feeds to efficiently produce the cultured organism (NRC, 2011; FAO, 2016).

Indispensable amino acids (IAAs) are essential nutrients required for normal growth and health. If proper amounts of all IAAs are not supplemented in aquaculture diets, organisms could potentially experience impaired protein synthesis, reduced weight gain, stunted growth, and a multitude of other health problems, ultimately resulting in death (Kaushik and Seiliez, 2010). The importance of accurate estimates of IAA requirements for aquaculture species has increased even more due to the need to replace fishmeal in aquafeeds with alternative protein feedstuffs (e.g., plant, animal and microbial meals), with the goal of reducing production costs and increasing the sustainability of the industry. However, most alternative protein feedstuffs may be deficient or imbalanced in one or more IAAs. Nonetheless, with refined estimates of IAA requirements for cultured species, the use of alternative ingredients in aquaculture diets can be maximized, allowing the development of nutritious and cost-effective feeds (NRC, 2011).

I.2 Amino Acids Requirements of Red Drum, *Sciaenops ocellatus*

Red drum (*Sciaenops ocellatus*) is a euryhaline sciaenid that is native to the Gulf of Mexico and Atlantic Ocean. This fish historically supported commercial and recreational fisheries for many decades; however, commercial overfishing provoked a decline in natural populations. Since then, research efforts related to the aquacultural production of this species for enhancement of wild populations as well as for food production were accelerated. Red drum for food markets have been reared to 1–2 kg in various culture systems, including earthen ponds, cages, net pens and raceways operating in flow-through or recirculating modes. Under favorable environmental conditions, red drum can be grown from a weight of 1 g to approximately 1 kg in 8–12 months (Gatlin, 2002).

Nutritious and cost-effective diets have been developed to support the aquacultural production of red drum based on their established requirements for some of the most critical nutrients and energy. The minimum dietary requirement of red drum for protein consisting of a balanced mixture of AAs has generally ranged from 350 to 450 g/kg of diet (Serrano et al., 1992). In addition, the metabolic protein requirements of red drum for maintenance and maximum growth were established at approximately 2 and 20 g digestible protein per kg of body weight per day, respectively (McGoogan and Gatlin, 1998).

Dietary requirements of red drum for most of the IAAs have been defined in previous studies using semi-purified diets consisting of a limited quantity of lyophilized red drum muscle (RDM) meal and supplemented with crystalline AAs, which have proven to be excellent test diets for quantitative AA studies with red drum (Gatlin, 2002). The total sulfur AA (cysteine + methionine) requirement was determined at 30.0 g/kg of dietary crude protein (CP) (Moon and Gatlin, 1991), the lysine requirement at 44.0 g/kg of CP (Craig and Gatlin, 1992), the threonine requirement at 23.0 g/kg of CP (Boren and Gatlin, 1995), the arginine requirement at 50.0 g/kg of CP (Barziza et al., 2000), the tryptophan requirement at 8.0 g/kg of CP (Pewitt et al., 2017), the histidine requirement at 16.0 g/kg of CP (Peachey and Gatlin, personal communication), and the total aromatic AA (phenylalanine + tyrosine) requirement at 60.0 g/kg of CP (Castillo et al., 2015).

Generally, quantification of IAA requirements is based on analysis of dose-response curves with weight gain used as a primary response criterion. The lowest level of EAA maximizing live weight gain is then identified as the minimum dietary requirement. Nonetheless, protein and AA deposition are also important response parameters that can contribute to a more robust and rational criteria (Encarnação et al., 2004). Moreover, blood and muscle AA levels are sometimes useful in corroborating EAA requirements because at sub-requirement intake levels, the serum or tissue content of the tested EAA should remain low until the requirement for that EAA is met and then increase to high levels when excessive amounts of the amino acid are fed (NRC, 2011).

I.3 Branched-chain Amino Acids: Leucine, Isoleucine and Valine

The branched-chain amino acids (BCAAs) are a group of IAAs composed of leucine (Leu), isoleucine (Ile) and valine (Val) that are characterized by having nonlinear and aliphatic side chains. BCAAs play important structural roles and are primarily deposited in body protein, notably in skeletal muscle; therefore, most proteins have a high amount of BCAAs, and these represent a significant proportion of the AAs consumed by animals. Due to their hydrophobicity, BCAAs are largely found in the interior core of globular proteins where their interactions with other similar AAs play a key role in determining the three-dimensional shapes of these proteins and, hence, their functions (Brosnan and Brosnan, 2006).

The BCAAs are increasingly recognized as anabolic nutrient signals, communicating the presence of an ingested protein-containing meal to peripheral tissues, stimulating insulin secretion by the β-cells of the pancreas and protein synthesis in muscle and adipose tissue through the mammalian target of rapamycin (mTOR) signalling pathway (Kim et al., 2013). In fish, it was confirmed that, as in mammals, BCAAs promote the hepatic expression of the insulin-like growth factor (IGF) gene in yellowtail (*Seriola quinqueradiata*) (Kawanago et al., 2014 and 2015) and the mTOR gene in rainbow trout (*Oncorhynchus mykiss*) (Lansard et al., 2011), confirming the role of BCAAs in fish growth. In addition, in other monogastric species (e.g., pigs and rats), BCAAs are known to be regulators of lipolysis, enhancing glucose consumption and utilization, promoting improved meat quality, benefitting embryo growth, enhancing intestinal development and intestinal AA transportation, and up-regulating innate and adaptive immune responses (Zhang et al., 2017).

The dietary requirements for Leu, Ile and/or Val have already been defined for several aquacultured fish species as summarized in the most recent National Research Council publication on aquatic species (NRC, 2011). Quantitative requirements for these three AAs have been reported for channel catfish *Ictalurus punctatus* (Leu 35.0 g/kg of CP; Ile 26.0 g/kg of CP; Val 30.0 g/kg of CP) (Wilson et al., 1980), common carp *Cyprinus carpio* (Leu 33.0 g/kg of CP; Ile 25.0 g/kg of CP; Val 36.0 g/kg of CP) (Nose, 1979), golden pompano *Trachinotus ovatus* (Leu 71.0-80.0 g/kg of CP; Val 46.0-47.0 g/kg of CP) (Tan et al., 2016; Huang et al., 2017), major Indian carp *Catla catla* (Leu 48.0 g/kg of CP; Val 31.0 g/kg of CP) (Zehra and Khan, 2014 and 2015), Nile tilapia *Oreochromis niloticus* (Leu 34.0 g/kg of CP; Ile 31.0 g/kg of CP; Val 28.0 g/kg of CP) (Santiago and Lovell, 1988; Gan et al., 2016), rainbow trout *O. mykiss* (Leu 23.0-29.0 g/kg of CP; Ile 15.0-28.0 g/kg of CP; Val 17.0-34.0 g/kg of CP) (Rodehutscord et al., 1997), red sea bream *Pagrus major* (Val 20.0 g/kg of CP) (Rahimnejad and Lee, 2013), rohu carp *Labeo rohita* (Leu 38.0-39.0 g/kg of CP; Ile 38.0-40.0 g/kg of CP; Val 37.5

g/kg of CP) (Khan and Abidi, 2007; Giri et al., 2015) and snout bream *Megalobrama amblycephala* (Leu 42.0-47.0 g/kg of CP; Val 37.0-39.0 g/kg of CP) (Ren et al., 2015a and 2015b).

I.4 Antagonism among Branched-chain Amino Acids

The metabolism of BCAAs is not restricted to the liver as for most IAA. In fact, the activity of BCAA transaminase in the liver is much lower than that in the skeletal muscle, kidney, heart and small intestine (Brosnan and Brosnan, 2006). In this transaminase reaction, the amino group is transferred from a BCAA to α-ketoglutarate (α-KG) to form glutamate and the respective branched-chain α-keto acid (BCKA) (Brosnan and Brosnan, 2006). It should be noted that all three BCAAs share the same common transporter for intestinal absorption and their oxidation is catalyzed by two common BCAA transaminase enzymes, and thus most organisms metabolize these three AAs using the same enzymatic system. Because of this, interactions between BCAAs are known to produce antagonistic effects in pigs, rats and humans, creating an imbalanced concentration of BCAAs in plasma and ultimately affecting growth. This has been mainly attributed to competitive inhibition during intestinal absorption and increased oxidation through BCKA dehydrogenase activation (Swendseid et al., 1965; Block and Harper, 1984; May et al., 1991; Matsumoto et al., 2014).

In fish, antagonism involving BCAAs has not been fully understood. Chance et al. (1964) observed that excess Leu and Ile in diets depressed growth of Chinook salmon (*O. tshawytscha*). In channel catfish, the antagonisms between BCAA did not occur when the three BCAAs met the dietary requirements (Robinson et al., 1984). However, an antagonistic effect of excess Leu on plasma and tissue BCAA concentrations was found in rainbow trout (Yamamoto et al., 2004), which was manifested as lower concentrations of Ile and Val in plasma when feeding diets formulated with high dietary Leu. Nonetheless, Choo et al. (1991) did not observe any effect of excess dietary Leu (134 g/kg of diet) on plasma Val and Ile concentrations in rainbow trout, concluding that growth depression and abnormal morphology of rainbow trout fed excess Leu diet did not result from antagonistic effects of BCAAs but from toxicity of the excess Leu itself.

I.5 Research Relevance and Ultimate Goals

Accurate estimates of all IAA requirements for aquaculture species are more significant than ever due to the need to replace fishmeal in aquafeeds with alternative protein feedstuffs that may be deficient or imbalanced in one or more IAAs. Moreover, red drum has proven to be a relevant species for the aquaculture industry with a steadily increasing global production. Therefore, the first objective of this dissertation was to determine red drum’s minimum dietary requirements for Leu, Ile and Val, completing red drum’s profile of quantitative IAA requirements.

On the other hand, interactions between BCAAs are known to produce antagonistic effects in pigs, rats and humans; however, in fish, antagonism involving BCAAs has not been fully understood. Therefore, the second objective of this dissertation was to elucidate the nature and potential impacts of the interactions among BCAAs in the aquaculture of red drum. This information could provide important physiological insights into the nutritional utilization of BCAAs in fish. In addition, it could potentially prevent impaired fish growth due to the imbalanced profile of BCAAs in some alternative protein feedstuffs (e.g., corn gluten meal has a high concentration of Leu [94.0 g/kg] relative to the concentration of other IAA like lysine [11.0 g/kg] and arginine [19.0 g/kg] [NRC, 2011]).

In conjunction, the goals of this dissertation will facilitate the use of alternative ingredients in aquaculture diets, allowing the development of nutritious and cost-effective feeds that will have an ideal AA profile for optimal performance of red drum.

CHAPTER II

DIETARY REQUIREMENTS FOR LEUCINE, ISOLEUCINE AND VALINE (BRANCHED-CHAIN AMINO ACIDS) BY JUVENILE RED DRUM

II.1 Introduction

Indispensable amino acids (IAAs) are essential nutrients required for normal growth and health. If proper amounts of all IAAs are not supplemented in aquaculture diets, organisms could potentially experience impaired protein synthesis, reduced weight gain, stunted growth, and a multitude of other health problems, ultimately resulting in death (Kaushik and Seiliez, 2010). The importance of accurate estimates of IAA requirements for aquaculture species has increased even more due to the need to replace fishmeal in aquafeeds with alternative protein feedstuffs (e.g., plant, animal and microbial meals), potentially reducing costs and increasing the sustainability of the industry. However, most alternative protein feedstuffs may be deficient or imbalanced in one or more IAAs. Nonetheless, with refined estimates of IAA requirements for cultured species, the use of alternative ingredients in aquaculture diets can be maximized, allowing the development of nutritious and cost-effective feeds (NRC, 2011).

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The branched-chain amino acids (BCAAs) are a group of IAAs composed of leucine (Leu), isoleucine (Ile) and valine (Val) that are largely found in the hydrophobic interior core of globular proteins, playing important structural roles and being primarily deposited in body protein, notably in skeletal muscle (NRC, 2011). In addition, BCAAs are increasingly recognized as an anabolic nutrient signal, communicating the presence of an ingested protein-containing meal to peripheral tissues, and promoting protein synthesis in fish muscle (Lansard et al., 2011; Kawanago et al., 2014 and 2015). Moreover, in other monogastric species (e.g., pigs and rats), BCAAs are known to be regulators of lipolysis, enhancing glucose consumption and utilization, promoting improved meat quality, benefitting embryo growth, enhancing intestinal development and intestinal AA transportation, and up-regulating innate and adaptive immune responses (Zhang et al., 2017).

With this in mind, the objective of the present study was to determine red drum's minimum dietary requirements for Leu, Ile and Val because of this species' relevance in aquaculture, and the need to complete its quantitative IAA requirements. This will facilitate the production of aquaculture feeds composed of alternative protein ingredients while maintaining an ideal amino acid (AA) profile for red drum's optimal performance.

II.2 Methods

II.2.1 Feeding trials

Fish husbandry and experimental protocols conducted in this study were approved by the Institutional Animal Care and Use Committee at Texas A&M

University (AUP IACUC 2016-0075). Three independent feeding trials were conducted at the Aquacultural Research and Teaching Facility at Texas A&M University, College Station, Texas, USA. Each feeding trial was focused on red drum's dietary requirement for Leu, Ile and Val, respectively. Juvenile red drum used in these feeding trials were obtained from the Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center operated by the Texas Parks and Wildlife Department in Lake Jackson, Texas, USA.

All three feeding trials were conducted in 38-L aquaria configured as a recirculating system (1 L/min), whereby waste water gravity-flowed to a settling chamber, then to a biological filter and was pumped through an ultraviolet light chamber and sand filter before being returned to the aquaria. Water quality was maintained within optimal levels for red drum. Salinity was maintained at 6–8 g/L by combining a synthetic seawater mixture (Fritz Industries, Mesquite, TX) with sodium chloride and well water, as recommended by manufacturer. Low-pressure electrical blowers provided aeration via air stones to maintain dissolved oxygen levels near air saturation (6.4 ± 0.02 mg/L). Water temperature was maintained at 27 ± 0.5°C by controlling ambient temperature with dual air-conditioning units. A 12 h light–12 h dark photoperiod was maintained with fluorescent lights controlled by automatic timers.

At the start of each feeding trial, juvenile red drum were stocked into aquaria to acclimate to experimental conditions for 1-wk, during which time a control diet was fed to apparent satiation. After the conditioning period, triplicate aquaria were randomly assigned to each dietary treatment (n=3). In the Leu trial, groups of 15 juvenile red drum were stocked into each aquarium at mean wet body weight (± standard deviation) of 1.42

± 0.19 g/fish. In the Ile trial, groups of 11 juvenile red drum were stocked into each aquarium at mean wet body weight (± standard deviation) of 3.68 ± 0.22 g/fish. In the Val trial, groups of 15 juvenile red drum were stocked into each aquarium at mean wet body weight (± standard deviation) of 2.83 ± 0.10 g/fish. Fish were fed twice daily at a rate approaching apparent satiation with pre-weighed rations based on a percentage of total fish weight per aquarium (50-60 g/kg of total body weight) and visual feeding cues. Fish in each aquarium were group-weighed every week and feed rations were adjusted accordingly. Each feeding trial continued for a total of 49 d.

II.2.2 Experimental diets

All experimental diets contained lyophilized red drum muscle (RDM) meal as intact protein (105 g/kg of dietary crude protein [CP]) and were supplemented with a premix of crystalline L-AAs to provide a total of 350 g/kg of CP in the diet (Table 1). The composition of the AA premix was adjusted to provide AA concentrations similar to that found in a diet with 350 g/kg of CP provided only by RDM, with the exclusion of the AA whose requirement was being tested (Leu, Ile or Val). Dietary lipid and dextrin were adjusted to provide 13.4 kJ of estimated digestible energy/g of diet (McGoogan and Gatlin, 1998). This diet composition has proven to make an excellent test diet for quantifying AA requirements of red drum because of its high palatability and nutritional quality, ensuring maximum growth potential of the experimental fish (Gatlin, 2002).

Table 1. Formulation and proximate composition of the basal diets (as dry g/kg).

Ingredients	**Leucine Trial**	**Isoleucine Trial**	**Valine Trial**
Red Drum Muscle Meal[1]	123	128	131
Crystalline Amino Acid Premix[2]	193	193	193
Dextrinized Starch[3]	35.0	35.0	35.0
Menhaden Oil[4]	81.5	82.0	74.0
Vitamin Premix[5]	30.0	30.0	30.0
Mineral Premix[5]	40.0	40.0	40.0
Carboxymethylcellulose[3]	10.0	10.0	10.0
Calcium Phosphate[6]	10.0	10.0	10.0
L-Glycine[7]	20.0	20.0	20.0
L-Aspartate/L-Glutamate Premix[7]	21.3	16.0	25.8
L-Leucine[7]	4.1	22.5	22.5
L-Isoleucine[7]	17.0	-	17.0
L-Valine[7]	19.8	19.8	-
Cellulose[3]	81.3	78.7	76.7
Proximate Composition[8]			
Crude Protein	375	372	369
Crude Lipid	104	105	112
Crude Ash	35.3	38.5	39.1

[1] From wild fish (Freeport, TX). Contained as dry g/kg: protein, 852; lipid, 140 (leucine trial); protein, 822; lipid, 142 (isoleucine trial); protein, 802; lipid, 184 (valine trial).

[2] Provided as crystalline L-amino acids (Ajinomoto North America, Inc.) as g/kg diet (in parenthesis is the total content of each amino acid in the diet): alanine, 18.0 (24.1); arginine, 16.1 (22.4); cysteine, 7.3 (8.3); glycine, 15.0 (41.2); histidine, 8.0 (10.3); lysine, 23.6 (33.5); methionine, 9.5 (12.7); phenylalanine, 15.5 (19.8); proline, 20.4 (24.1); serine, 22.8 (26.6); taurine, 8.9 (10.1); threonine, 12.3 (17.3); tryptophan, 4.2 (5.6); tyrosine, 11.3 (14.6).

[3] USB Corporation, Cleveland, OH.

[4] Special Select, Omega Protein Corporation, Houston, TX.

[5] Same as (Moon and Gatlin, 1991).

[6] Sigma-Aldrich, St. Louis, MO.

[7] Ajinomoto North America, Inc., Itasca, IL.

[8] Means of two replicate analyses.

The amount of Leu, Ile and Val contributed by lyophilized RDM in each of the basal diets was quantified by ultra-performance liquid chromatography (UPLC - Acquity system®, Waters™, Milford, MA) according to the procedures previously described by Castillo et al. (2015), and were found to contain Leu, Ile and Val at 8.90 g/kg, 5.00 g/kg and 6.80 g/kg of dry diet, respectively. For the Leu feeding trial, five experimental diets were prepared by supplementing the basal diet with crystalline L-Leu to provide increments of 40.0 g/kg of dry diet (9.0 [basal], 13.0, 17.0, 21.0, 25.0 and 29.0 g/kg of dry diet). For the Ile feeding trial, five experimental diets were prepared by supplementing the basal diet with crystalline L-Ile to provide increments of 3.0 g/kg of dry diet (5.0 [basal], 8.0, 11.0, 14.0, 17.0 and 20.0 g/kg of dry diet). Finally, for the Val feeding trial, six experimental diets were prepared by supplementing the basal diet with crystalline L-Val to provide increments of 1.20 g/kg of dry diet (6.80 [basal], 8.00, 9.20, 10.4, 11.6, 12.8 and 14.0 g/kg of dry diet). All inclusion values were chosen based on previously documented BCAA requirements of other carnivorous fish species (NRC, 2011). In addition, the experimental diets were kept isonitrogenous by adjusting the inclusion level of an aspartate/glutamate premix, resulting in a final CP value of 370 g/kg.

All diets were prepared by mechanically mixing the dry ingredients in a V-mixer. The dry mixture was then blended with oil and water and pressure extruded using an industrial mixer with a meat grinding attachment with a 3-mm die. Resulting pellets were dried on ventilated mesh drying racks for 24 h using forced air at 25°C (McGoogan and Gatlin, 1998). Additionally, AA concentrations in the diets were analyzed via UPLC

(Acquity system, Waters Corporation, Milford, MA) and the commercial kit MassTrak (Castillo et al., 2015). The MassTrak kit uses precolumn derivatization of AAs with a 6-aminoquenolyl-N-hydroxysuccinimidyl carbamate tag (AccQTag), followed by reversed-phase UPLC on a C18 column (1.7 µm; 2.1 x 150 mm) and UV detection at 260 nm. Analyzed concentrations of Leu, Ile and Val in subsamples of all experimental diets from each feeding trial confirmed the targeted values with standard deviations no higher than 0.05% of diet.

II.2.3 Sampling, performance indices and laboratory analyses

After each feeding trial, final fish weight and percent survival in each aquarium were determined. Prior to sample collection, all fish to be sampled at each time period were euthanized via tricainemethane sulphonate overdose (MS-222 at 300 mg/L, Western Chemical, Inc., Ferndale, WA). Then, three fish per aquarium were randomly sampled at 16 h after the last feeding (postprandial time point). Total body weight, along with fillets (muscle), liver, and intraperitoneal fat (IPF) weights were recorded for three fish per aquarium for calculation of body condition indices as follows: muscle ratio (g fillet weight/100 g body weight), hepatosomatic index (HSI) (g liver weight/100 g body weight) and IPF ratio (g IPF weight/100 g body weight). Additionally, whole-body pooled samples (n=3 fish/tank) were taken and stored at −20°C until analyzed for proximate composition using established methodology: CP (N factor=6.25) (AOAC, 2005), crude fat (Folch et al., 1957), and ash (AOAC, 1990). In addition, growth performance and feed efficiency indicators were computed as follows: relative weight gain ([g final weight−initial weight/g initial weight] × 100), feed efficiency ratio (FE=g weight gain/g dry feed intake) and protein retention efficiency (PRE=[final body protein−initial body protein] × 100/total protein fed). Finally, to quantify the postprandial concentration of BCAAs in plasma, three fish were sampled 6 h after the last feeding and blood was collected from the caudal vasculature with the use of heparinized needles, centrifuged (2000 x *g*, 10 min) for plasma separation and stored at -80°C. Quantification of plasma-free Leu, Ile and Val was done with the use of UPLC analyses in an Acquity UPLC system with integrated TUV detector and MassTrak AAA Solutions Kit (Waters Corporation). All samples were deproteinized with 1.5 mol/L $HClO_4$ (9552-05; JT Baker), neutralized with 2 mol/L K_2CO_3 (P5833; Sigma Chemical), and centrifuged at 12,000 x g for 5 min. Supernatant fluid was filtered through 0.2-µm polycarbonate syringe filters before derivatization (Castillo et al., 2015).

II.2.4 Statistical analyses

Normality and homoscedasticity assumptions were confirmed prior to any statistical analysis. All evaluated variables were subjected to an analysis of variance (ANOVA) to determine if the inclusion levels of Leu, Ile and/or Val significantly ($P<0.05$) affected the observed responses; in addition, a follow-up trend analysis using orthogonal polynomial contrasts was performed to determine if the significant effects were linear and/or quadratic. The quantitative requirement for Leu and Ile was estimated with a quadratic broken-line regression analysis; whereas, the Val requirement was estimated with a linear broken-line regression analysis. These analyses are based on a two straight-line, single break point model with respect to the response criteria, providing a break point estimate with a 95% confidence interval (CI), interpreted as the nutrient requirement above which there is no significant change in the response variable (Robbins et al., 2006). The broken-line model which best fit each set of data was chosen based on R^2, mean square error, and *P*-value of the parameter estimates. All statistical analyses were conducted using SAS Software (v. 9.4).

II.3 Results

II.3.1 Dietary leucine requirement

Incremental levels of Leu in the diet significantly affected weight gain, feed efficiency and protein retention (ANOVA, *P*<0.001), where positive linear and quadratic trends were found (Table 2). Red drum's performance improved as the concentration of Leu increased in the diet, but it quickly reached a plateau where higher concentrations of Leu did not seem to affect the performance parameters. On subjecting the percent weight gain data to a quadratic broken-line regression model, a breakpoint was found at 15.7 ± 0.17 g/kg (± 95% CI) of dry diet (42.4 g/kg of dietary CP), which represents the minimum Leu requirement for maximum growth of juvenile red drum (Figure 1). By using the protein retention data as the response criterion, the Leu requirement was estimated at 16.3 ± 1.8 g/kg (± 95% CI) of dry diet.

In line with the growth performance responses, muscle ratio was significantly affected by the incremental levels of Leu in the diet (ANOVA, *P*<0.001); however, HSI and IPF ratio were not significantly affected (Table 3). Regarding the postprandial concentration of BCAAs in plasma, incremental levels of dietary Leu did not affect the concentration of Leu in plasma, but did significantly affect the concentration of Ile and Val (ANOVA, $P<0.01$), where a negative linear trend was found (Table 3).

Table 2. Growth, feed efficiency, protein retention, and survival of red drum fed graded amounts of leucine for 7 weeks.[A]

Dietary Leu, g/kg	Mean Initial Weight, g	Mean Final Weight, g	Relative Weight Gain, %	Feed Efficiency Ratio	Protein Retention, %	Survival, %
9.0	1.47 ± 0.13	4.93 ± 0.35	237 ± 7.50	0.50 ± 0.01	19.7 ± 0.67	100 ± 0.00
13.0	1.52 ± 0.06	10.8 ± 0.28	610 ± 13.5	0.84 ± 0.02	35.9 ± 1.40	97.8 ± 2.22
17.0	1.40 ± 0.10	10.7 ± 0.82	658 ± 26.2	0.88 ± 0.04	39.7 ± 1.35	88.9 ± 4.44
21.0	1.38 ± 0.11	10.8 ± 0.90	679 ± 10.9	0.92 ± 0.01	40.4 ± 1.10	93.3 ± 3.85
25.0	1.38 ± 0.07	11.4 ± 0.45	721 ± 12.6	0.91 ± 0.02	39.9 ± 0.36	85.3 ± 4.74
29.0	1.33 ± 0.05	10.5 ± 0.28	665 ± 41.0	0.89 ± 0.03	39.7 ± 1.72	88.9 ± 2.22
ANOVA (Pr>F[B])	-	<0.001	<0.001	<0.001	<0.001	0.08
Linear Trend (Pr>F)	-	<0.001	<0.001	<0.001	<0.001	-
Quadratic Trend (Pr>F)	-	<0.001	<0.001	<0.001	<0.001	-

[A] Values represent mean ± standard error of three replicate aquaria.

[B] Significance probability associated with the F-statistic.

Figure 1. Relationship between weight gain of juvenile red drum and dietary leucine level as described by a quadratic broken-line regression model. The break point estimate is interpreted as the leucine level above which there is no significant change in the response criteria.

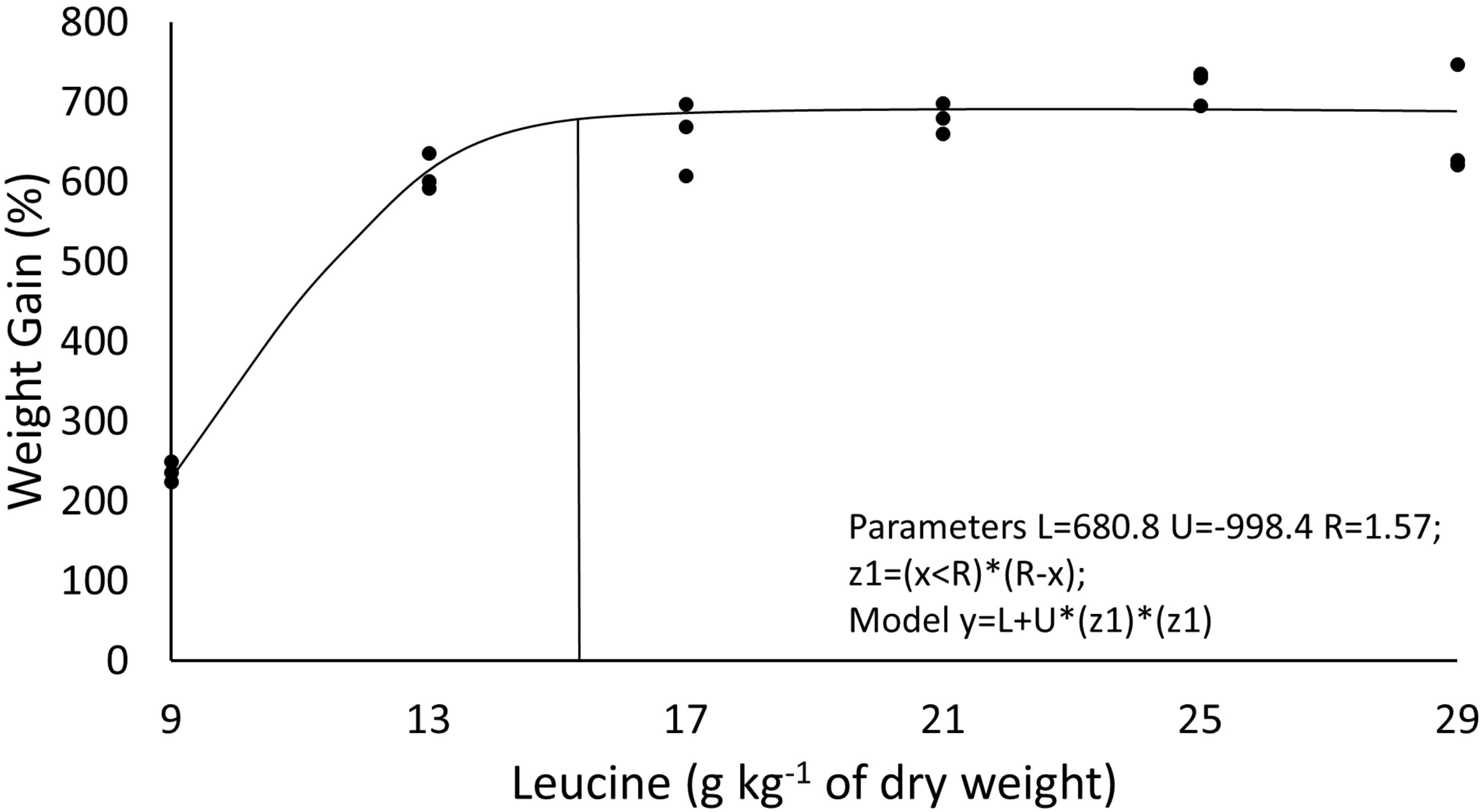

Table 3. Body condition indices and postprandial (6 h after last feeding) concentration of branched-chain amino acids in plasma of red drum fed graded amounts of leucine for 7 weeks.[A]

Dietary Leu, g/kg	**Body Condition Indices**			**BCAA Concentration in Plasma, mMol/mL**		
	HSI	**Muscle Ratio**	**IPF Ratio**	**Leucine**	**Isoleucine**	**Valine**
9.0	2.97 ± 0.27	19.3 ± 0.63	0.27 ± 0.05	29.9 ± 2.27	47.1 ± 6.46	107 ± 15.3
13.0	3.39 ± 0.25	28.8 ± 2.26	0.21 ± 0.04	31.2 ± 1.70	33.3 ± 1.67	60.5 ± 5.37
17.0	3.08 ± 0.09	30.6 ± 2.79	0.18 ± 0.03	35.2 ± 4.99	43.3 ± 8.63	99.9 ± 19.9
21.0	3.39 ± 0.17	28.6 ± 2.61	0.14 ± 0.03	32.5 ± 4.23	19.4 ± 1.42	36.8 ± 3.54
25.0	3.37 ± 0.10	31.5 ± 2.35	0.23 ± 0.04	26.3 ± 1.38	16.1 ± 1.21	33.7 ± 7.30
29.0	3.61 ± 0.27	29.7 ± 0.51	0.19 ± 0.07	33.6 ± 4.32	19.7 ± 2.84	35.3 ± 5.93
ANOVA (Pr>F[B])	0.33	<0.001	0.77	0.54	0.01	0.003
Linear Trend (Pr>F)	-	<0.001	-	-	0.003	0.001
Quadratic Trend (Pr>F)	-	0.001	-	-	0.37	0.56

[A] Values represent mean ± standard error of three fish from each of three replicate aquaria.

[B] Significance probability associated with the F-statistic.

II.3.2 Dietary isoleucine requirement

Incremental levels of Ile in the diet significantly affected weight gain, feed efficiency and protein retention (ANOVA, $P<0.001$), where positive linear and quadratic trends were found (Table 4). As in the Leu trial, red drum's performance improved as the concentration of Ile increased in the diet, but it quickly reached a plateau where higher concentrations of Ile did not seem to affect the performance parameters. On subjecting the percent weight gain data to a quadratic broken-line regression model, a breakpoint was found at 11.1 ± 0.23 g/kg (± 95% CI) of dry diet (30.0 g/kg of dietary CP), which represents the minimum Ile requirement for maximum growth of juvenile red drum (Figure 2). By using the protein retention data as the response criterion, the Leu requirement was estimated at 10.7 ± 1.8 g/kg (± 95% CI) of dry diet.

Muscle ratio was significantly affected by the incremental levels of Ile in the diet (ANOVA, $P<0.001$); however, HSI and IPF ratio were not significantly affected (Table 5). Regarding the postprandial concentration of BCAAs in plasma, incremental levels of dietary Ile significantly affected the plasma concentration of Leu, Ile and Val (ANOVA, $P<0.05$); however, only a positive linear trend in the plasma concentration of Ile in response to the incremental levels of dietary Ile was found (Table 5).

Table 4. Growth, feed efficiency, protein retention, and survival of red drum fed graded amounts of isoleucine for 7 weeks.[A]

Dietary Ile, g/kg	Mean Initial Weight, g	Mean Final Weight, g	Relative Weight Gain, %	Feed Efficiency Ratio	Protein Retention, %	Survival, %
5.0	3.65 ± 0.02	11.9 ± 1.06	227 ± 28.3	0.41 ± 0.03	17.8 ± 1.10	100 ± 0.00
8.0	3.80 ± 0.10	22.5 ± 0.33	491 ± 10.8	0.76 ± 0.02	30.4 ± 1.46	97.0 ± 3.03
11.0	3.74 ± 0.14	25.8 ± 2.02	592 ± 63.0	0.75 ± 0.04	29.2 ± 2.89	81.8 ± 6.06
14.0	3.59 ± 0.16	25.9 ± 0.73	625 ± 44.9	0.76 ± 0.03	29.6 ± 1.56	77.7 ± 9.09
17.0	3.60 ± 0.11	23.8 ± 2.22	560 ± 47.3	0.73 ± 0.01	29.5 ± 0.95	81.8 ± 5.24
20.0	3.70 ± 0.05	24.4 ± 1.59	558 ± 44.8	0.74 ± 0.05	29.0 ± 2.20	84.9 ± 8.02
ANOVA (Pr>F[B])	-	<0.001	<0.001	<0.001	0.003	0.06
Linear Trend (Pr>F)	-	<0.001	<0.001	0.001	0.004	-
Quadratic Trend (Pr>F)	-	<0.001	<0.001	0.001	0.003	-

[A] Values represent mean ± standard error of three replicate aquaria.

[B] Significance probability associated with the F-statistic.

Figure 2. Relationship between weight gain of juvenile red drum and dietary isoleucine level as described by a quadratic broken-line regression model. The break point estimate is interpreted as the isoleucine level above which there is no significant change in the response criteria.

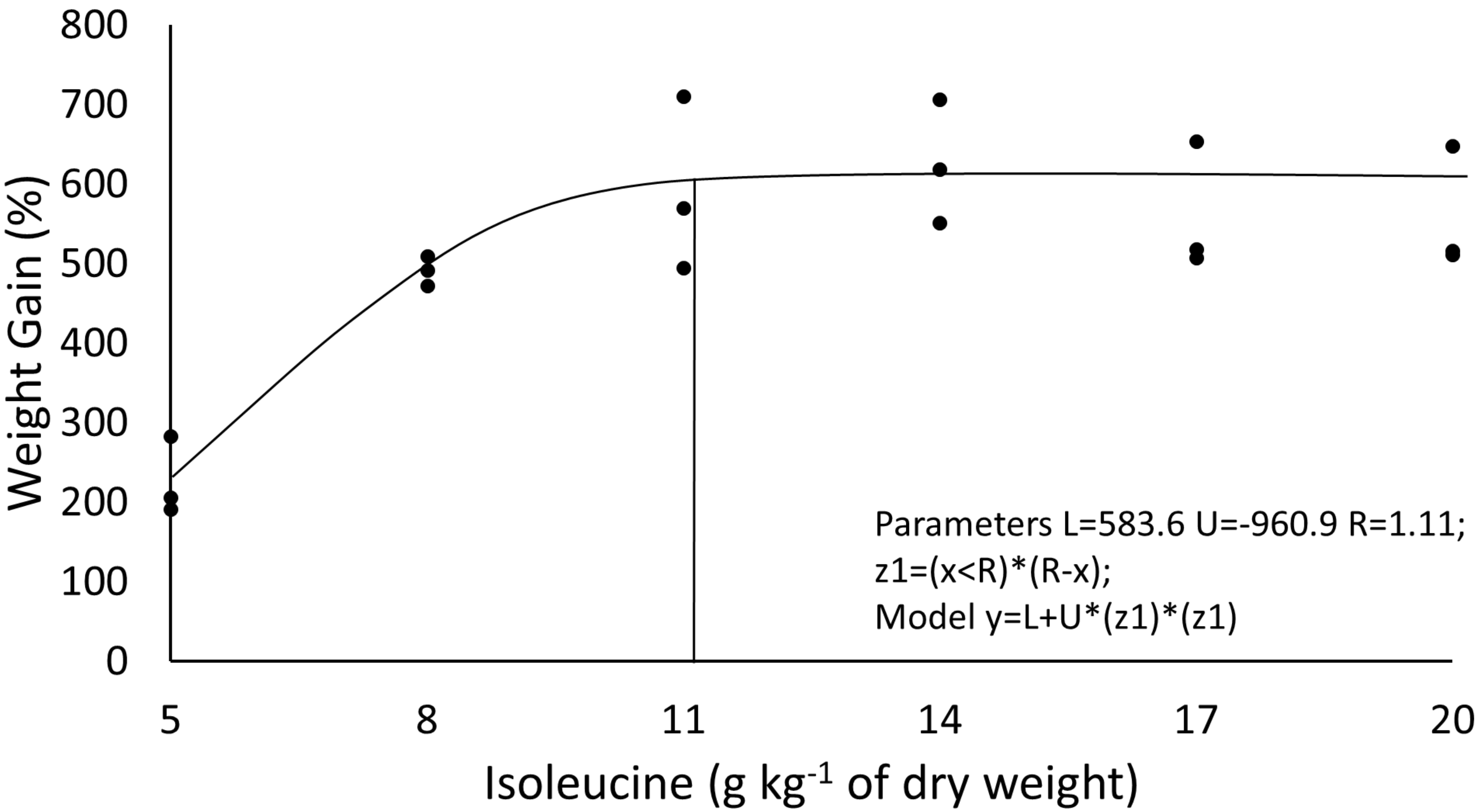

Table 5. Body condition indices and postprandial (6 h after last feeding) concentration of branched-chain amino acids in plasma of red drum fed graded amounts of isoleucine for 7 weeks.[A]

Dietary Ile, g/kg	Body Condition Indices			BCAA Concentration in Plasma, mMol/mL		
	HSI	Muscle Ratio	IPF Ratio	Leucine	Isoleucine	Valine
5.0	2.72 ± 0.32	26.0 ± 1.79	0.27 ± 0.03	62.8 ± 13.1	5.59 ± 3.28	72.3 ± 12.0
8.0	3.64 ± 0.03	32.7 ± 1.02	0.44 ± 0.14	92.4 ± 12.1	11.7 ± 3.92	88.7 ± 10.6
11.0	3.46 ± 0.42	33.0 ± 0.07	0.31 ± 0.01	133 ± 28.1	31.5 ± 8.11	114 ± 19.5
14.0	4.11 ± 0.87	33.4 ± 0.06	0.33 ± 0.06	44.8 ± 5.84	12.7 ± 2.20	47.9 ± 5.94
17.0	3.46 ± 0.21	33.0 ± 1.70	0.35 ± 0.05	77.7 ± 13.5	36.6 ± 7.31	89.5 ± 14.5
20.0	3.34 ± 0.06	34.7 ± 0.25	0.39 ± 0.08	84.9 ± 24.6	62.5 ± 19.9	96.0 ± 16.9
ANOVA (Pr>F[B])	0.40	0.002	0.64	0.03	0.001	0.04
Linear Trend (Pr>F)	-	<0.001	-	0.88	<0.001	0.64
Quadratic Trend (Pr>F)	-	0.02	-	0.39	0.19	0.90

[A] Values represent mean ± standard error of three fish from each of three replicate aquaria.

[B] Significance probability associated with the F-statistic.

II.3.3 Dietary valine requirement

Incremental levels of Val in the diet significantly affected weight gain, feed efficiency and protein retention (ANOVA, $P<0.001$), where positive linear and quadratic trends were found (Table 6). Red drum’s performance significantly improved as the concentration of Val increased in the diet; however, contrary to the Ile and Leu trials, the plateaus of the performance responses were found towards the end of the curve, at higher dietary Val concentrations. On subjecting the percent weight gain data to a linear broken-line regression model, a breakpoint was found at 12.4 ± 0.6 g/kg (± 95% CI) of dry diet (33.5 g/kg of dietary CP), which represents the minimum Val requirement for maximum growth of juvenile red drum (Figure 3). By using the protein retention data as the response criterion, the Val requirement was estimated at 11.9 ± 0.5 g/kg (± 95% CI) of dry diet.

In line with the growth performance responses, all body condition indices evaluated were significantly affected by the incremental levels of Val in the diet (ANOVA, $P<0.01$), where positive linear and quadratic trends were found (Table 7). On the other hand, the postprandial concentration of BCAAs in plasma was not significantly affected by dietary Val levels (Table 7).

Table 6. Growth, feed efficiency, protein retention, and survival of red drum fed graded amounts of valine for 7 weeks.[A]

Dietary Val, g/kg	Mean Initial Weight, g	Mean Final Weight, g	Relative Weight Gain, %	Feed Efficiency Ratio	Protein Retention, %	Survival, %
6.8	2.90 ± 0.06	5.04 ± 0.18	74.0 ± 3.79	0.19 ± 0.02	6.18 ± 0.69	68.6 ± 5.88
8.0	2.77 ± 0.03	7.15 ± 0.25	159 ± 11.3	0.37 ± 0.04	15.4 ± 1.47	80.0 ± 10.2
9.2	2.85 ± 0.10	10.7 ± 0.35	278 ± 19.7	0.60 ± 0.01	25.3 ± 1.00	93.3 ± 3.85
10.4	2.72 ± 0.07	14.3 ± 1.46	424 ± 40.1	0.75 ± 0.02	31.5 ± 0.45	82.2 ± 9.69
11.6	2.74 ± 0.07	16.8 ± 0.95	516 ± 39.7	0.81 ± 0.05	36.5 ± 1.89	79.3 ± 11.8
12.8	2.91 ± 0.03	19.9 ± 0.12	585 ± 7.40	0.87 ± 0.03	40.2 ± 1.30	80.6 ± 9.68
14.0	2.86 ± 0.07	19.2 ± 1.32	574 ± 43.0	0.87 ± 0.02	40.1 ± 0.65	93.3 ± 0.00
ANOVA (Pr>F[B])	-	<0.001	<0.001	<0.001	<0.001	0.15
Linear Trend (Pr>F)	-	<0.001	<0.001	<0.001	<0.001	-
Quadratic Trend (Pr>F)	-	0.03	0.01	<0.001	<0.001	-

[1] Values represent mean ± standard error of three replicate aquaria.

[2] Significance probability associated with the F-statistic.

Figure 3. Relationship between weight gain of juvenile red drum and dietary valine level as described by a linear broken-line regression model. The break point estimate is interpreted as the valine level above which there is no significant change in the response criteria.

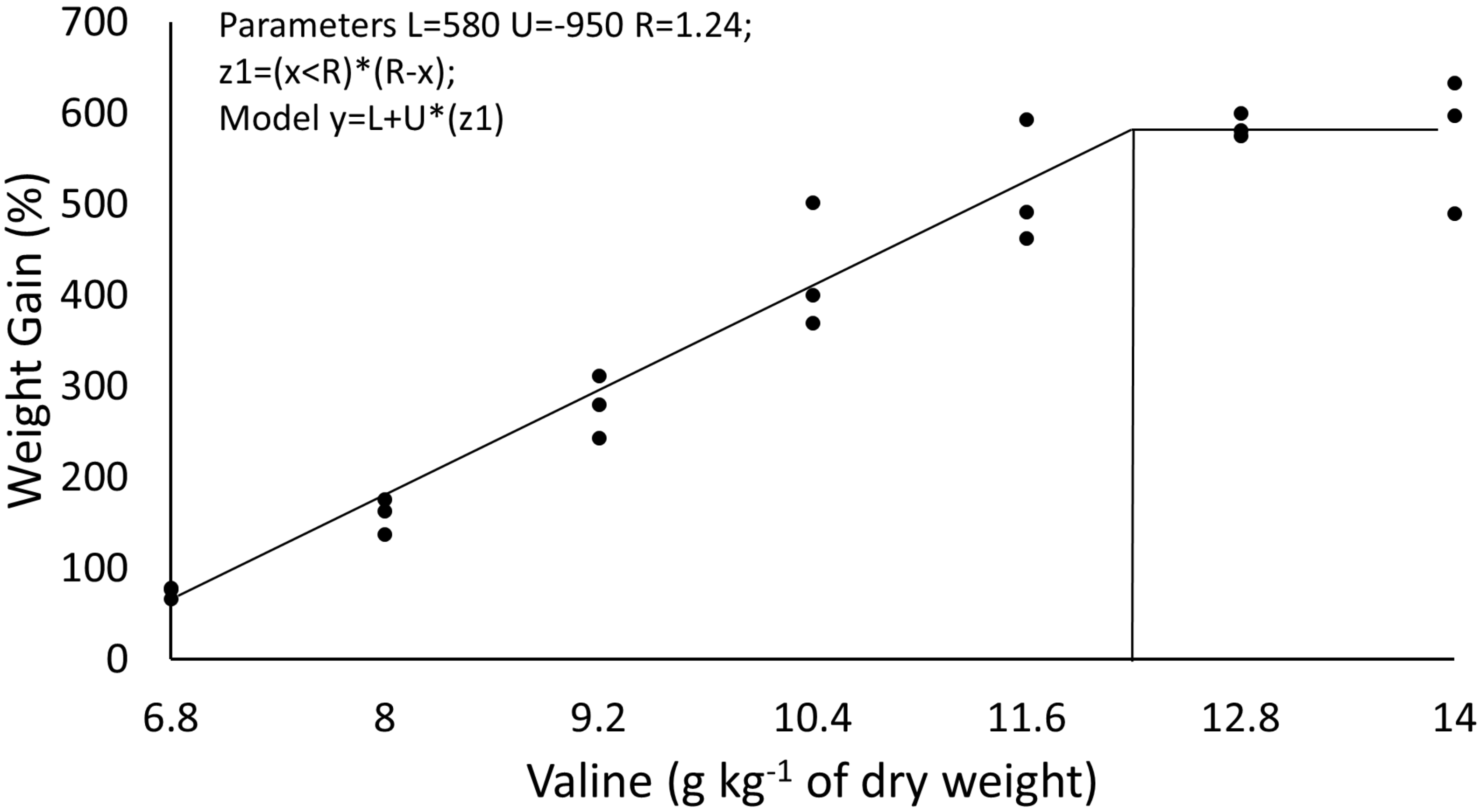

Table 7. Body condition indices and postprandial (6 h after last feeding) concentration of branched-chain amino acids in plasma of red drum fed graded amounts of valine for 7 weeks.[A]

Dietary Val, g/kg	Body Condition Indices			BCAA Concentration in Plasma, mMol/mL		
	HSI	Muscle Ratio	IPF Ratio	Leucine	Isoleucine	Valine
6.8	1.30 ± 0.24	23.4 ± 1.40	0.02 ± 0.01	39.3 ± 5.31	27.1 ± 7.81	47.6 ± 7.97
8.0	1.50 ± 0.11	25.1 ± 0.84	0.01 ± 0.01	59.8 ± 7.33	43.8 ± 2.94	42.2 ± 6.99
9.2	2.37 ± 0.15	28.0 ± 1.57	0.20 ± 0.11	54.3 ± 6.87	37.2 ± 5.35	46.3 ± 7.78
10.4	2.99 ± 0.15	29.3 ± 0.90	0.42 ± 0.13	51.2 ± 21.1	25.5 ± 6.41	43.0 ± 6.46
11.6	3.24 ± 0.17	31.5 ± 0.83	0.43 ± 0.08	64.6 ± 13.2	21.0 ± 5.30	48.2 ± 4.28
12.8	3.20 ± 0.33	31.6 ± 0.79	0.39 ± 0.03	44.4 ± 6.69	26.7 ± 7.59	43.8 ± 4.53
14.0	3.25 ± 0.07	29.4 ± 0.40	0.18 ± 0.01	56.2 ± 8.35	29.6 ± 9.79	41.4 ± 6.75
ANOVA (Pr>F[B])	<0.001	<0.001	0.003	0.10	0.52	0.51
Linear Trend (Pr>F)	<0.001	<0.001	0.002	-	-	-
Quadratic Trend (Pr>F)	0.01	0.004	0.002	-	-	-

[A] Values represent mean ± standard error of three fish from each of three replicate aquaria.

[B] Significance probability associated with the F-statistic.

II.4 Discussion

With the goal of facilitating the production of aquaculture feeds composed of alternative protein ingredients with an ideal AA profile for red drum's optimal performance, this study was focused on determining red drum's minimum dietary requirements for Leu, Ile and Val (BCAAs). Incremental dietary levels of Leu, Ile and Val significantly affected all growth and feed efficiency responses evaluated, confirming the importance of establishing the minimum dietary concentration of BCAAs necessary to support optimal growth of red drum. Generally, quantification of IAA requirements is based on an analysis of dose-response curves with weight gain used as a response criterion. However, some authors suggest that protein deposition may be a more robust and rational criterion to estimate AA requirements; especially after finding that, in rainbow trout, estimated requirements based on protein deposition were higher than those based on weight gain (Encarnação et al., 2004). In the present study, it was reassuring to find that the Leu, Ile and Val requirements estimated based on red drum's weight gain and protein deposition responses were very similar.

The estimated dietary requirements for Leu, Ile and Val in juvenile red drum fell within the higher end of the range of BCAA requirements estimated in other fish species when compared as percentage of dietary CP (Nose, 1979; Wilson et al., 1980; Santiago and Lovell, 1988; NRC, 2011). Even though the BCAA requirements for other commonly cultured marine carnivorous fish have not been determined, estimated red drum's requirements are comparable to those reported for some other freshwater/anadromous carnivorous fish like rainbow trout, Atlantic salmon and Pacific salmon (Rodehutscord et al., 1997; NRC, 2011). It is important to note that experimental conditions and methodologies (e.g., diet composition, fish size, statistical approach, etc.) may influence estimated IAA requirements (De Silva et al., 2000; Simmons et al., 1999). Regarding diet composition, it is important to prepare palatable and highly digestible semi-purified diets to ensure that the fish will reach their maximum growth potential while precisely determining the minimum IAA requirement (Kaushik and Seiliez, 2010). The experimental diets prepared in this study contained lyophilized RDM which compared to other purified ingredients (e.g., casein or gelatin), has been shown to improve the palatability and protein utilization of experimental diets, and therefore feed intake and growth performance of red drum (Gatlin, 2002). Likewise, to limit the influence of unbalanced AA ratios, an AA premix with ideal AA ratios was prepared based on the AA pattern found in RDM and included in the experimental diets.

The analysis of postprandial concentrations of AAs in plasma is considered a valuable tool to complement the conclusions obtained from growth performance responses in studies of AA requirements. At sub-requirement intake levels, the plasma concentration of the tested AA should remain low until the requirement is met and then increase to high levels when excessive amounts of the AA are fed (Cowey, 1994). Interestingly, there was no clear effect of dietary Leu, Ile and Val on the postprandial (6 h after feeding) concentrations of BCAAs in plasma that could point to an optimal requirement level. Dietary Leu and Val did not have any significant effect on the concentration of Leu and Val in plasma, respectively. Meanwhile, dietary Ile did significantly affect the concentration of Ile in plasma, where an increase was observed at the estimated requirement level (11.0 g/kg); but then, the concentration decreased again when dietary Ile increased to 14.0 g/kg. There are some other instances where the postprandial concentration of AAs in plasma has not been a reliable indicator of IAA requirements (Kaushik and Luquet, 1979; Walton et al., 1984b; Kim et al., 1992; Castillo et al., 2015). The validity of these observations seems to be linked to the nature of the AA tested, interactions among different AAs, and time elapsed between meal intake and blood sampling (Mambrini and Kaushik, 1995).

Regarding body condition indices, all dietary BCAAs had a significant effect on muscle ratio, which was clearly correlated with the growth performance and protein retention responses. On the other hand, dietary Leu and Ile did not significantly affect the HSI and IPF ratio; however, with increasing levels of dietary Val both HSI and IPF ratio significantly increased. Based on the growth performance responses evaluated, the basal level of dietary Val used in this study appeared to be more metabolically limiting than the basal levels of dietary Leu and Ile used. Therefore, this could explain why only increasing dietary levels of Val significantly affected the HSI and IPF ratio in juvenile red drum.

Various AA deficiency signs have been documented in different fish species. For example, salmonid species exhibited cataracts when given a diet deficient in methionine (Poston et al., 1977), as well as histidine (Waagbø et al., 2010); whereas, tryptophan deficiency was reported to cause scoliosis and derangement of mineral metabolism in certain salmonids (Walton et al., 1984a; Akiyama et al., 1986). In the case of red drum, recent studies showed that fish fed deficient levels of tryptophan did not exhibit any visual signs of deficiency (Pewitt et al., 2017); however, fish fed deficient levels of histidine did exhibit formation of cataracts (Peachey and Gatlin, personal communication). In the case of the present study, besides reduced growth and feed efficiency, no other deficiency signs were observed in red drum fed the lowest dietary levels of Leu, Ile and Val.

It should be noted that all three BCAAs share the same common transporter for intestinal absorption and their oxidation is catalyzed by two common BCAA transaminase enzymes, and thus most organisms metabolize these three AAs using the same enzymatic system (Brosnan and Brosnan, 2006). Because of this, interactions between BCAAs are known to produce antagonistic effects in pigs, rats and humans; creating an imbalanced concentration of BCAAs in plasma and ultimately affecting growth. This has been mainly attributed to competitive inhibition during intestinal absorption and increased oxidation through branched-chain α-keto acid dehydrogenase kinase (BCKDH) (Brosnan and Brosnan, 2006). In fish, antagonism involving BCAAs has not been fully assessed (NRC, 2011). An antagonist effect of excess Leu on plasma and tissue BCAA concentrations was found in rainbow trout by Yamamoto et al. (2004), reporting lower concentrations of Ile and Val in plasma when feeding diets formulated to low Ile:Leu and Val:Leu ratios. However, Choo et al. (1991) did not observe any effect of excess dietary Leu on plasma Val and Ile concentrations in rainbow trout, where even excessive dietary Leu concentrations (134 g/kg of diet) did not depress the concentrations of Val and Ile in plasma, liver and muscle. In the present study, weight gain was not affected by the highest dietary concentrations of Leu, Ile and Val; however, increasing levels of dietary Leu significantly reduced the concentration of Ile and Val in plasma, where a significantly negative linear trend was found. Similar results have been found in humans (Swendseid et al., 1965; Matsumoto et al., 2014) and rats (Block and Harper, 1984; May et al., 1991), as well as rainbow trout (Yamamoto et al., 2004); this is possibly due to the role of Leu as a promoter of BCAA catabolism by activating BCKDH (Paxton and Harris, 1984). Antagonistic effects among BCAAs in red drum will be further explored in the next chapter of this dissertation, where higher dietary concentrations and different ratios of BCAAs were tested.

CHAPTER III

ELUCIDATING ANTAGONISTIC EFFECTS AMONG BRANCHED-CHAIN AMINO ACIDS IN JUVENILE RED DRUM

III.1 Introduction

All three branched-chain amino acids (BCAAs) share the same common transporter for intestinal absorption and their oxidation is catalyzed by two common BCAA transaminase enzymes, and thus most organisms metabolize these three amino acids (AAs) using the same enzymatic system. Because of this, interactions between BCAAs are known to produce antagonistic effects in pigs, rats and humans, creating an imbalanced concentration of BCAAs in plasma and ultimately affecting growth. This has been mainly attributed to competitive inhibition during intestinal absorption and increased oxidation through branched-chain α–keto acid dehydrogenase kinase (BCKDH) activation (Swendseid et al., 1965; Block and Harper, 1984; May et al., 1991; Matsumoto et al., 2014).

In fish, antagonism involving BCAAs has not been fully understood. Chance et al. (1964) observed that excess leucine (Leu) and isoleucine (Ile) in diets depressed growth of Chinook salmon (*Oncorhynchus tshawytscha*). In channel catfish (*Ictalurus punctatus*), an excess of Leu and Ile depressed growth of fish fed diets deficient in BCAAs, but not of fish fed diets where BCAAs met the minimum dietary requirements (Robinson et al., 1984). However, an antagonistic effect of excess Leu on plasma and tissue BCAAs concentration was found in rainbow trout (*O. mykiss*) (Yamamoto et al., 2004), reporting lower concentrations of Ile and Val in plasma when feeding diets formulated with high dietary Leu. Nonetheless, Choo et al. (1991) did not observe any effect of excess dietary Leu (134 g/kg of diet) on plasma valine (Val) and Ile concentrations in rainbow trout.

The objective of the present study was to elucidate the nature and potential impacts of the interactions among BCAAs in the aquaculture of red drum. This information could provide important physiological insights into the nutritional utilization of BCAAs in fish; in addition, it could potentially prevent impaired fish growth due to the imbalanced profile of BCAAs in some alternative protein feedstuffs (e.g., corn gluten meal has a high concentration of Leu [94.0 g/kg] relative to the concentration of other IAA like lysine [11.0 g/kg] and arginine [19.0 g/kg] [NRC, 2011]).

III.2 Methods

II.2.1 Feeding trials

Fish husbandry and experimental protocols conducted in this study were approved by the Institutional Animal Care and Use Committee at Texas A&M University (AUP IACUC 2016-0075). Two independent feeding trials were conducted at the Aquacultural Research and Teaching Facility at Texas A&M University, College Station, Texas, USA. First, a preliminary trial (Leu trial) was focused on characterizing the possible antagonistic effect of excess Leu in the diet of red drum. Secondly, a follow-up and more comprehensive feeding trial (BCAAs trial) was focused on elucidating antagonistic effects among all three BCAAs. Juvenile red drum used in these feeding trials were obtained from the Sea Center Texas Marine Aquarium, Fish Hatchery and

Nature Center operated by the Texas Parks and Wildlife Department in Lake Jackson, Texas, USA.

Both feeding trials were conducted in 38-L aquaria configured as a recirculating system (1 L/min), whereby waste water gravity-flowed to a settling chamber, then to a biological filter and was pumped through an ultraviolet light chamber and sand filter before being returned to the aquaria. Water quality was maintained within optimal levels for red drum. Salinity was maintained at 6–8 g/L by combining a synthetic seawater mixture (Fritz Industries, Mesquite, TX) with sodium chloride and well water, as recommended by the manufacturer. Low-pressure electrical blowers provided aeration via air stones to maintain dissolved oxygen levels near air saturation (6.4 ± 0.02 mg/L). Water temperature was maintained at 27 ± 0.5°C by controlling ambient temperature with dual air-conditioning units. A 12 h light–12 h dark photoperiod was maintained with fluorescent lights controlled by automatic timers.

At the start of each feeding trial, juvenile red drum were stocked into aquaria to acclimate to experimental conditions for 1-wk, during which time a control diet was fed to apparent satiation. After the conditioning period, triplicate aquaria were randomly assigned to each dietary treatment (n=3). In the Leu trial, groups of 15 juvenile red drum were stocked into each aquarium at mean wet body weight (± standard deviation) of 3.51 ± 0.11 g/fish. Whereas, in the BCAAs trial, groups of 12 juvenile red drum were stocked into each aquarium at mean wet body weight (± standard deviation) of 4.56 ± 0.27 g/fish. The experimental fish were fed twice daily at a rate approaching apparent satiation with pre-weighed rations based on a percentage of total fish weight per aquarium (50-60 g/kg of total body weight) and visual feeding cues. Fish in each aquarium were group-weighed every two weeks and feed rations were adjusted accordingly. The preliminary Leu trial continued for a total of 30 d, while the BCAAs trial continued for a total of 45 d.

II.2.2 Experimental diets

For both feeding trials, a semi-purified control diet (Table 8) containing 430 g/kg of crude protein (CP) was prepared by combining lyophilized red drum muscle (RDM) meal as an intact protein source (105 g/kg of dietary CP) and supplementing with a premix of crystalline L-AAs simulating the AA pattern found in RDM, while keeping the levels of Leu, Ile and Val at the minimum dietary requirement levels for red drum's optimal performance (16.0, 11.0 and 12.0 g/kg of dry diet, respectively), which were quantified and reported in the previous chapter of this dissertation. Dietary lipid and dextrin were adjusted to provide 13.4 kJ of estimated digestible energy/g of diet (McGoogan and Gatlin, 1998). This diet composition has proven to make an excellent test diet for studies on AA nutrition for red drum because of its high palatability and nutritional quality, ensuring maximum growth potential of the experimental fish (Gatlin, 2002).

Table 8. Formulation and proximate composition of the control diet for both the Leu trial and the BCAAs trial.

Ingredients	**Dry weight, g/kg**
Red Drum Muscle Meal[1]	128
Crystalline Amino Acid Premix[2]	193
Dextrinized Starch[3]	350
Menhaden Oil[4]	82.0
Vitamin Premix[5]	30.0
Mineral Premix[5]	40.0
Carboxymethylcellulose[3]	10.0
Calcium Phosphate[6]	10.0
L-Glycine[7]	20.0
L-Aspartate/L-Glutamate Premix[7]	96.5
L-Leucine[7]	7.50
L-Isoleucine[7]	6.00
L-Valine[7]	6.80
Cellulose[3]	25.3
Proximate Composition[8]	
Crude Protein	428
Crude Lipid	101
Crude Ash	34.1

[1] From wild fish (Freeport, TX). Contained as dry g/kg: protein, 822; lipid, 142.

[2] Provided as crystalline L-amino acids (Ajinomoto North America, Inc.) as g/kg diet (in parenthesis is the total content of each amino acid in the diet): alanine, 18.0 (24.1); arginine, 16.1 (22.4); cysteine, 7.3 (8.3); glycine, 15.0 (41.2); histidine, 8.0 (10.3); lysine, 23.6 (33.5); methionine, 9.5 (12.7); phenylalanine, 15.5 (19.8); proline, 20.4 (24.1); serine, 22.8 (26.6); taurine, 8.9 (10.1); threonine, 12.3 (17.3); tryptophan, 4.2 (5.6); tyrosine, 11.3 (14.6).

[3] USB Corporation, Cleveland, OH.

[4] Special Select, Omega Protein Corporation, Houston, TX.

[5] Same as (Moon and Gatlin, 1991).

[6] Sigma-Aldrich, St. Louis, MO.

[7] Ajinomoto North America, Inc., Itasca, IL.

[8] Means of two replicate analyses.

In addition to the control diet, for the preliminary Leu trial, two experimental diets were prepared by (1) removing the supplementation of Leu (Leu deficient diet – 9.00 g/kg of dry diet) and by (2) supplementing an excess of Leu (62.0 g/kg of dry diet). Additionally, for the BCAAs trial, six experimental diets were prepared by supplementing the control diet with (1) an excess of Leu (62.0 g/kg of dry diet), (2) an excess of Ile (44.0 g/kg of dry diet), (3) an excess of Val (50.0 g/kg of dry diet), (4) an excess of Leu and Ile (62.0 and 44.0 g/kg of dry diet), (5) an excess of Ile and Val (44.0 and 50.0 g/kg of dry diet), and (6) an excess of Leu and Val (62.0 and 50.0 g/kg of dry diet). Excess levels of BCCAs corresponded to 200% of the amount that would be found in a diet with 350 g/kg CP provided by RDM. All the experimental diets were kept isonitrogenous by adjusting the inclusion level of an aspartate/glutamate premix.

All diets were prepared by mechanically mixing the dry ingredients in a V-mixer. The dry mixture was then blended with oil and water and pressure extruded using an industrial mixer with a meat grinding attachment with a 3-mm die. Resulting pellets were dried on ventilated mesh drying racks for 24 h using forced air at 25°C (McGoogan and Gatlin, 1998). Additionally, AA concentrations in the diets were analyzed via UPLC (Acquity system, Waters Corporation, Milford, MA) and the commercial kit MassTrak (Castillo et al., 2015). The MassTrak kit uses precolumn derivatization of AAs with a 6-aminoquenolyl-N-hydroxysuccinimidyl carbamate tag (AccQTag), followed by reversed-phase UPLC on a C18 column (1.7 µm; 2.1 x 150 mm) and UV detection at 260 nm. Analyzed concentrations of Leu, Ile and Val in subsamples of all experimental diets from each feeding trial confirmed the targeted values with standard deviations no higher than 0.05% of diet.

II.2.3 Sampling, performance indices and laboratory analyses

Prior to sample collection, all fish to be sampled at each time period were euthanized via tricainemethane sulphonate overdose (MS-222 at 300 mg/L, Western Chemical, Inc., Ferndale, WA). In the Leu trial, final fish weight and percent survival in each aquarium were determined. Afterward, relative weight gain ([g final weight−initial weight/g initial weight] × 100) and feed efficiency ratio (FE=g weight gain/g dry feed intake) were computed. To quantify the postprandial concentration of BCAAs in the plasma of fish fed the experimental diets for 30 days, three fish per aquarium were randomly sampled at 2, 6 and 24 h after the last feeding and blood was collected from the caudal vasculature with the use of heparinized needles, centrifuged (2000 x *g*, 10 min) for plasma separation and stored at -80°C. Additionally, to evaluate the short-term effect of the diets, a separate group of fish were fed the experimental diets only once and the postprandial concentration (2 h after feeding) of BCAAs in plasma was quantified. Lastly, muscle samples were collected to determine the concentration of BCAAs deposited as body protein in the muscle of fish fed the experimental diets for 30 days.

In the BCAAs trial, final fish weight and percent survival in each aquarium were determined. Then, total body weight, along with fillets (muscle), liver, and intraperitoneal fat (IPF) weights were recorded from three fish per aquarium for calculation of body condition indices as follows: hepatosomatic index (HSI) (g liver weight/100 g body weight) and IPF ratio (g IPF weight/100 g body weight). Afterward, whole-body pooled samples (n=3 fish/tank) were taken and stored at −20°C until analyzed for proximate composition using established methodology: CP (N factor=6.25) (AOAC, 2005), crude fat (Folch et al., 1957), and ash (AOAC, 1990). In addition, growth performance and feed efficiency indicators were computed as follows: relative weight gain ([g final weight−initial weight/g initial weight] × 100), feed efficiency ratio (FE=g weight gain/g dry feed intake) and protein retention efficiency (PRE=[final body protein−initial body protein] × 100/total protein fed). Lastly, to quantify the postprandial concentration of BCAAs in the plasma of fish fed the experimental diets for 45 d, three fish per aquarium were randomly sampled at 1 and 4 h after the last feeding and blood was collected from the caudal vasculature with the use of heparinized needles, centrifuged (2000 x *g*, 10 min) for plasma separation and stored at -80°C. In addition, muscle samples were collected at 4 h after feeding to determine the postprandial concentration of free pool BCAAs in muscle.

Quantification of plasma-free, muscle-free and muscle-bound Leu, Ile and Val was done with the use of UPLC analyses in an Acquity UPLC system with integrated TUV detector and MassTrak AAA Solutions Kit (Waters Corporation). All samples were deproteinized with 1.5 mol/L $HClO_4$ (9552-05; JT Baker), neutralized with 2 mol/L K_2CO_3 (P5833; Sigma Chemical), and centrifuged at 12,000 x g for 5 min. Supernatant fluid was filtered through 0.2-µm polycarbonate syringe filters before derivatization (Castillo et al., 2015).

II.2.4 Statistical analyses

Normality and homoscedasticity assumptions were confirmed prior to any statistical analysis. In the Leu trial, all response criteria were subjected to an analysis of variance (ANOVA) with significance set at $P<0.05$. Significant ANOVA were followed by Tukey's HSD post hoc multiple comparison test to detect significant differences among the three dietary treatments. Meanwhile, in the BCAAs trial, a full factorial model (2^3 with missing cells) was used to detect significant ($P<0.05$) main effects and two-way interactions. Dunnett's test was used to compare the significant main effects and/or two-way interactions against the control group. All statistical analyses were conducted using SAS Software (v. 9.4).

III.3 Results

After 30 d, weight gain was significantly affected in fish that were fed both an excess and a deficiency of Leu when compared to those fed the control diet; although, growth impairment in the deficient group was more severe. Meanwhile, feed efficiency was only significantly affected in fish fed the deficient diet. In contrast, survival was not significantly affected by any of the dietary treatments (Table 9).

Table 9. Growth performance, feed efficiency and survival of juvenile red drum fed the experimental diets with different inclusion levels of leucine (Leu) for 30 days.[1]

	Control	**Leu Excess**	**Leu Deficient**	**Pr>F**[2]	**SEM**[3]
Initial Mean Weight, g	3.44	3.34	3.62	0.84	0.12
Final Mean Weight, g	17.0^{A}	14.7^{B}	7.78^{C}	<0.001	0.39
Relative Weight Gain, %	394^{A}	339^{B}	115^{C}	<0.001	11.0
Feed Efficiency Ratio	0.91^{A}	0.90^{A}	0.69^{B}	0.03	0.05
Survival, %	80.0	88.9	95.6	0.31	6.54

[1] Values represent means of three replicate groups (n=3).

[2] Significance probability associated with the F-statistic. Different superscript letters within a row indicate significant (P<0.05) differences as evaluated by Tukey's test.

[3] Pooled mean standard error.

In the BCAAs trial, fish fed the control diet presented the highest weight gain after 45 d. However, weight gain was only significantly affected in fish fed an excess of Leu, alone or in combination (Leu+Ile and Leu+Val). Neither an excess of Ile nor Val significantly affected red drum's growth. Feed efficiency and HSI were not significantly affected by the different dietary treatments; however, a significant interaction among Leu+Ile increased the IPF ratio when compared to the control group. Lastly, survival was significantly higher in diets with an excess of BCAAs when compared to the control group (Table 10).

The postprandial (2 and 6 h after feeding) concentration of BCAAs in plasma of red drum at the end of the Leu trial are presented in Table 11. Fish fed a diet deficient in Leu showed a lower concentration of plasma Leu and a higher concentration of plasma Ile and Val than the control group, although these differences were significant only at the 2 h after feeding sampling point. In contrast, when compared to the control group, fish fed an excess of Leu presented a significantly higher concentration of plasma Leu, but the plasma Ile and Val levels were not significantly affected. In addition, the postprandial concentration of BCAAs in fish fed the experimental diets only once were very similar (Table 12) to those of fish fed the diets for 30 consecutive days. Lastly, there was not a significant effect of an excess or deficiency of Leu on the concentration of BCAAs deposited as body protein in the muscle of fish fed the experimental diets for 30 d (Table 13).

Table 10. Growth performance, feed utilization, body condition indices and survival of juvenile red drum fed the control diet and the experimental diets with an excess of branched-chain amino acids (BCAA) for 45 days.[1]

	BCAA Excess Incorporation							**Pr>F[2]**	**PSE[3]**
	Control	**Leu**	**Ile**	**Val**	**Leu+Ile**	**Ile+Val**	**Leu+Val**		
Initial Mean Weight, g	4.44	4.75	4.58	4.55	4.61	4.54	4.38	0.80	0.17
Final Mean Weight, g	31.0	26.3	28.1	29.7	26.2	25.1	25.5	0.09	1.48
Relative Weight Gain, %	597	455*	511	554	469	454	481	0.02	29.0
Feed Efficiency Ratio	0.85	0.81	0.86	0.86	0.87	0.83	0.80	0.06	0.02
Hepatosomatic Index	3.14	3.18	2.91	2.88	3.41	2.87	2.95	0.49	0.21
Intraperitoneal Fat Ratio	0.30	0.49	0.34	0.24	0.37*	0.25	0.37	0.03	0.03
Survival (%)	86.1	100	100	97.2	97.2*	97.2*	91.7*	0.03	2.10

[1] Values represent means of three replicate groups (n=3).

[2] An (*) indicates significant ($P<0.05$) main effects and/or two-way interactions.

[3] Pooled standard error.

Table 11. Branched-chain amino acids levels in plasma (nMol/mL) of juvenile red drum fed the experimental diets with different inclusion levels of leucine (Leu) for 30 days.[1]

	Control	**Leu Excess**	**Leu Deficient**	**Pr>F[2]**	**PSE[3]**
			2 h After Feeding		
Leucine	61.7^{B}	411^{A}	3.96^{C}	<0.001	15.5
Isoleucine	25.7^{B}	27.2^{B}	74.7^{A}	<0.001	7.40
Valine	85.8^{B}	82.3^{B}	134^{A}	0.03	13.3
			6 h After Feeding		
Leucine	67.5^{B}	885^{A}	15.3^{B}	<0.001	0.12
Isoleucine	37.1	40.8	96.4	0.15	23.2
Valine	106	115	163	0.50	36.1

[1] Values represent means of three replicate groups (n=3).

[2] Significance probability associated with the F-statistic. Different superscript letters within a row indicate significant (P<0.05) differences as evaluated by Tukey's test.

[3] Pooled standard error.

Table 12. Branched-chain amino acids levels in plasma (nMol/mL) of juvenile red drum fed the experimental diets with different inclusion levels of leucine (Leu) only once.[1]

	Control	**Leu Excess**	**Leu Deficient**	**Pr>F[2]**	**PSE[3]**
			2 h After Feeding		
Leucine	70.0^{B}	536^{A}	20.0^{C}	<0.001	8.52
Isoleucine	37.5^{B}	33.5^{B}	100^{A}	0.01	11.4
Valine	116^{B}	101^{B}	175^{A}	0.02	16.4

[1] Values represent means of three replicate groups (n=3).

[2] Significance probability associated with the F-statistic. Different superscript letters within a row indicate significant (P<0.05) differences as evaluated by Tukey's test

[3] Pooled standard error.

Table 13. Concentration of branched-chain amino acids deposited as body protein in muscle (g/100 g) of juvenile red drum fed the experimental diets with different inclusion levels of leucine (Leu) for 30 days.[1]

	Control	**Leu Excess**	**Leu Deficient**	**Pr>F[2]**	**PSE[3]**
Leucine	7.42	7.85	6.51	0.37	0.67
Isoleucine	4.22	4.50	3.68	0.34	0.38
Valine	4.81	5.08	4.19	0.35	0.43

[1] Values represent means of three replicate groups (n=3).

[2] Significance probability associated with the F-statistic.

[3] Pooled standard error.

The postprandial (1 and 4 h after feeding) levels of BCAAs in plasma of red drum at the end of the BCAAs trial are presented in Table 14. Fish fed an excess of Leu showed a significantly higher concentration of plasma Leu than the control group at 4 h after feeding; although, at 1 h after feeding, this effect was not found. Nonetheless, in fish fed an excess of Leu+Ile/Val, a significantly higher concentration of plasma Leu at both sampling points was found when compared to the control group. The same results were found in fish fed an excess of Ile and/or Val, where an increase in the concentration of plasma Ile and/or Val was found. In contrast, an excess of dietary Leu, Ile and/or Val did not significantly affect the plasma levels of the BCAAs that were not supplemented in excess in the diet. In addition, a significant interaction among Ile+Val was found, where the concentration of plasma Ile and Val was at least two-fold higher than in any of the other treatments. Lastly, fish fed the different experimental diets presented very similar trends in the concentration of free pool BCAAs in muscle (Table 15) when compared with the postprandial concentration of BCAAs in plasma.

Table 14. Branched-chain amino acids levels in plasma (nMol/mL) of juvenile red drum fed the control diet and the experimental diets with an excess of branched-chain amino acids (BCAA) for 45 days.[1]

	BCAA Excess Incorporation							**Pr>F[2]**	**PSE[3]**
	Control	**Leu**	**Ile**	**Val**	**Leu+Ile**	**Ile+Val**	**Leu+Val**		
				1 h After Feeding					
Leucine	64.3	66.0	92.2	58.0	210*	75.3	253*	<0.001	35.1
Isoleucine	41.3	72.5	89.2	30.5	74.9	153*	33.6	0.002	24.2
Valine	65.7	24.4	57.3	90.5	27.8	183*	187*	<0.001	33.8
				4 h After Feeding					
Leucine	46.9	481*	107	70.1	474	313	462	<0.001	62.8
Isoleucine	55.0	121	508	110	428	992*	43.4	<0.001	72.1
Valine	71.9	89.3	80.4	788	238	1466*	711	<0.001	110

[1] Values represent means of three replicate groups (n=3).

[2] An (*) indicates significant (P<0.05) main effects and/or two-way interactions.

[3] Pooled standard error.

Table 15. Free pool of branched-chain amino acids in muscle (nMol/mL) of juvenile red drum fed the control diet and the experimental diets with an excess of branched-chain amino acids (BCAA) for 45 days.[1]

	BCAA Excess Incorporation							**Pr>F**[2]	**PSE**[3]
	Control	**Leu**	**Ile**	**Val**	**Leu+Ile**	**Ile+Val**	**Leu+Val**		
				4 h After Feeding					
Leucine	5.19	26.1	6.92	13.0	24.8	9.78	17.0*	0.002	4.19
Isoleucine	4.86	13.1	17.9*	7.57	16.3	18.9	4.23	<0.001	2.68
Valine	5.73	9.35	15.3	27.3*	14.4	33.3	18.3	0.003	4.96

[1] Values represent means of three replicate groups (n=3).

[2] An (*) indicates significant ($P<0.05$) main effects and/or two-way interactions.

[3] Pooled standard error.

III.4 Discussion

Interactions between BCAAs are known to produce antagonistic effects in pigs, rats and humans, creating an imbalanced concentration of BCAAs in plasma and ultimately affecting growth (Swendseid et al., 1965; Block and Harper, 1984; May et al., 1991; Matsumoto et al., 2014). However, in fish, antagonism involving BCAAs has not been fully characterized or understood. In the present study with red drum, growth at the end of both feeding trials was significantly affected by an excess of Leu, alone or in combination (Leu+Ile and Leu+Val), confirming an antagonistic effect due to the imbalanced concentration of BCAAs in the diet.

To attain the goals of this study, it was important to select appropriate dietary levels of BCAAs. A control diet was prepared by keeping dietary Leu, Ile and Val at minimum requirement levels for optimal performance of red drum. Meanwhile, excess levels of BCCAs were incorporated into the experimental diets by doubling (200%) the amount that would be found in a diet with 350 g/kg CP provided by RDM. At the end of both feeding trials, the postprandial concentration of plasma BCAAs in fish fed the control diet did not change between the different sampling points (1, 2, 4 and 6 h after feeding); whereas, when an excess of BCAAs was present in the diet, the plasma concentration of these AAs was significantly higher than in the control group and kept increasing over time. These results suggest that the dietary BCAAs levels incorporated into the experimental diets were appropriate for uncovering potential antagonistic effects in red drum.

In other fish species, several studies have been done in the past trying to elucidate potential antagonistic effects among BCAAs with some inconsistent results.

Chance et al. (1964) observed that an excess of Leu (60 g/kg of diet) and Ile (30 g/kg of diet) depressed growth of Chinook salmon. Similarly, Nose (1979) noted that growth of common carp was suboptimal when an excess of Ile (28.6 g/kg) was included in the diet. In the present study with red drum, it was noticeable that fish fed an excess of Ile grew less than the control group, although not significantly. Nonetheless, it is possible that by increasing the dietary concentration of Ile or by prolonging the duration of the feeding trial, an excess of Ile could have affected growth of red drum significantly, as was reported for salmon and carp. In channel catfish, an excess of dietary Leu (16.8 g/kg of diet) and Ile (12.4 g/kg), but not Val (14.2 g/kg), depressed growth of fish fed diets deficient in BCAAs, but not of fish fed diets where BCAAs met the minimum dietary requirements (Robinson et al., 1984). However, the excess levels of dietary BCAAs used in the study with catfish were lower than the supplementation levels used in other studies where an antagonism was found. For example, Hughes et al. (1984) reported that antagonistic effects were clearly observed in lake trout fed an excess of Leu (50.0 g/kg of diet), and Val supplementation to the excess Leu diets relieved the depression in growth and feed utilization. Choo et al. (1991) observed that an excess of Leu (134 g/kg of diet) resulted in growth depression and abnormal morphology of rainbow trout. Similarly, Yamamoto et al. (2004) concluded that antagonistic effects due to excess Leu (57.0 g/kg of diet) occurred in rainbow trout, and enrichment with Ile and Val relieved the adverse effects on growth performance and amino acid metabolism. Finally, Han et al. (2014) and Wang et al. (2017) found that Japanese flounder growth was affected by an excess of Leu (51.0 g/kg of diet) but only when an excess of Ile (44.0 g/kg) or Val (25.0 g/kg) was present in the diet as well.

Excess levels of dietary Leu are known to result in an imbalanced concentration of plasma BCAAs, where reduced levels of plasma Ile and Val have been found in humans (Swendseid et al., 1965; Matsumoto et al., 2014) and rats (Block and Harper, 1984; May et al., 1991). Authors have attributed this response to competitive inhibition during intestinal absorption and to the role of Leu as a promoter of the catabolism of BCAAs by activating BCKDH, the enzyme that catabolizes BCKAs (Paxton and Harris, 1984). Similar results were found in rainbow trout by Yamamoto et al. (2004), reporting lower concentrations of Ile and Val in plasma when feeding diets formulated with an excess of Leu. In the present study with red drum, the whole profile of plasma AAs levels was obtained; however, only the plasma BCAAs were significantly affected by the dietary treatments. Fish fed an excess of Leu, Ile and/or Val presented a significantly higher concentration of the BCAAs in plasma that were supplemented in excess. However, despite what has been found in other species, the concentration of BCAAs in plasma that were not supplemented in the diet in excess was not significantly affected when compared to the control group. Similarly, Choo et al. (1991) did not observe any effect of excess dietary Leu on plasma Val and Ile concentrations in rainbow trout, where even excessive dietary Leu concentrations (134 g/kg of diet) did not depress the levels of Val and Ile in plasma, liver and muscle. In addition, Han et al. (2014) and Wang et al. (2017) did not find a significant effect of excess dietary Leu on plasma Ile and Val levels of Japanese flounder. As was suggested by Yamamoto et al. (2004), it is possible that inconsistent results among different studies may be due to differences among the experimental diets. For example, crystalline AAs, which are absorbed from the digestive tract more rapidly than AAs from intact proteins (Williams et al., 2001), could be masking the effects of excess dietary Leu on the concentration of AAs in plasma. The use of practical diets to evaluate the effects of excess BCAAs on red drum should be explored for further understanding of this issue.

Although the postprandial levels of BCAAs in plasma of red drum did not indicate that an excess of Leu blocked the intestinal absorption or promoted the catabolism of Ile and/or Val, a competitive inhibition cannot be discarded. The activity of the BCAA transaminase enzyme is known to be high in muscle, kidney, heart and small intestine. In this transaminase reaction, the amino group is transferred from a BCAA to α-ketoglutarate (α-KG) to form glutamate and the respective BCKA (Brosnan and Brosnan, 2006). In fact, large amounts of BCAAs are metabolized in the intestine. In addition, after food ingestion, glutamate is known to be converted into BCAAs, proline and alanine (Adeva et al., 2011). Therefore, the postprandial concentration of AAs in plasma could be a misleading parameter in trying to identify an antagonistic effect among BCAAs. As an alternative, the postprandial concentration of α-KG in plasma could provide valuable insight into the postprandial metabolism of BCAAs and, therefore, a better understanding of the effects of excess BCAAs in the diet.

Additional mechanisms by which excess BCAAs may be affecting fish performance have been reported. In rainbow trout, Choo et al. (1991) concluded that adverse effects caused by an excess of Leu resulted from toxicities because, despite the significant growth impairment, those authors did not observe any alteration of plasma and muscle Val and Ile concentrations. Studies with rats and humans have found that the accumulation of toxic BCAA metabolites may trigger mitochondrial dysfunction. After the transamination of BCAAs, the liver then oxidizes BCKAs liberating increased levels of propionyl CoA which is converted to succinyl CoA and thereby floods the TCA cycle with intermediates. Increased levels of propionyl CoA and succinyl CoA allosterically inhibit citrate synthase which in turn inhibits glucose oxidation and its utilization causing glucose intolerance (Guillet et al., 2009; Newgard et al., 2009). In fact, increased levels of plasma BCAAs have been reported to lead to insulin resistance or type 2 diabetes mellitus (Adeva et al., 2011; Sheriff et al., 2014).

On a separate note, remarkable results were found in the Ile+Val treatment. Even though neither an excess of Ile nor Val significantly affected red drum's growth performance, when both an excess of Ile+Val was present in the diet, significant growth impairment was observed. Moreover, the concentration of plasma Ile and Val was at least two times higher than in any of the other treatments. It is possible to hypothesize that a saturation of both Ile and Val in plasma impeded the catabolism of Leu, increasing the concentration of plasma Leu. Consequently, the concentration of plasma Val and Ile increased even more because there was less BCKA derived from Leu to activate the catabolism of BCAAs by BCKDH. Future studies should take a closer look into the effects of excess Ile+Val to further explore this hypothesis.

Additional and interesting results were obtained from the preliminary Leu trial. First, it was observed that red drum fed a diet deficient in Leu presented a significantly higher concentration of plasma Ile and Val than the control group; possibly confirming the role of Leu in promoting the catabolism of Ile and Val. Secondly, the postprandial concentration of plasma BCAAs was very similar in fish fed the experimental diets only once versus fish fed the experimental diets for 30 consecutive d. Therefore, there is no sign of a short-term (30 d) adaptation to high or low levels of dietary Leu in red drum. Finally, even though there was a significant growth impairment when fish were fed an excess of Leu, these fish still performed significantly better (three-fold) than fish fed a diet deficient in Leu, suggesting that there is high plasticity in the digestion and/or metabolism of BCAAs in red drum.

Finally, besides growth of red drum and plasma AA levels, other responses were also evaluated. Notably, fish fed an excess of Leu presented a significantly higher IPF ratio than the control group. This response is common when a dietary insufficiency (AA imbalance) alters nutrient retention (NRC, 2011). In addition, the HSI, considered a liver function indicator, was not significantly affected by the dietary treatments. Nonetheless, in Japanese flounder, HSI decreased significantly when both high Leu and Ile were present in the diet (Wang et al., 2017). Lastly, survival was significantly lower in the control group. This has been previously observed in studies with red drum, where fish fed the best performing diets exhibit a more aggressive behavior increasing the mortality rate.

CHAPTER IV

OVERALL CONCLUSIONS

Juvenile red drum were responsive to dietary BCAA supplementation with fish fed the basal diets exhibiting significantly reduced weight gain and feed utilization. Analyses of weight gain data using a broken-line regression model estimated the minimum Leu, Ile and Val requirements for maximum growth of juvenile red drum to be 15.7 ± 1.7 g/kg (± 95% CI), 11.1 ± 2.3 g/kg and 12.4 ± 0.6 g/kg of dry diet, respectively. These results will facilitate the production of aquaculture feeds composed of alternative protein ingredients while maintaining an ideal AA profile for red drum's optimal performance.

Growth performance of red drum was significantly affected by an excess of dietary Leu, alone or in combination (Leu+Ile and Leu+Val), confirming an antagonistic effect due to the imbalanced concentration of BCAAs in the diet. In addition, the postprandial concentration of plasma Leu, Ile and/or Val was significantly higher in fish fed an excess of Leu, Ile and/or Val, respectively. Nonetheless, postprandial levels of BCAAs in plasma did not indicate that an excess of Leu blocked the intestinal absorption or promoted the catabolism of Ile and/or Val in red drum, as has been reported in other species. Ultimately, these results are a step forward in understanding the nature of the antagonistic effects among BCAAs in fish, and will potentially prevent impaired performance due to the imbalanced profile of BCAAs in some alternative protein feedstuffs.

REFERENCES

Adeva, M.M., Calvino, J., Souto, G., Donapetry, C. (2011). Insulin resistance and the metabolism of branched-chain amino acids in humans. Amino Acids, 43, 171-181.

Akiyama, T., Murai, T., Mori, K. (1986). Role of tryptophan metabolites in inhibition of spinal deformity of chum salmon fry caused by tryptophan deficiency. Nippon Suisan Gakk, 52, 1255–1259.

AOAC (1990). Official Methods of Analysis, 15th ed. Association of Official Analytical Chemists, Arlington, USA.

AOAC (2005). Official Methods of Analysis. 18th ed. Association of Official Analytical Chemists. Arlington, USA.

Barziza, D.E., Buentello, J.A., Gatlin III, D.M. (2000). Dietary arginine requirement of juvenile red drum (*Sciaenops ocellatus*) based on weight gain and feed efficiency. The Journal of Nutrition, 130, 1796-1799.

Block, K.P., Harper, A.E. (1984). Valine metabolism in vivo: Effects of high dietary levels of leucine and isoleucine. Metabolism, 33, 559-566.

Boren, R.S., Gatlin III, D.M. (1995). Dietary threonine requirement of juvenile red drum *Sciaenops ocellatus*. Journal of the World Aquaculture Society, 26, 279-283.

Brosnan, J., Brosnan, M. (2006). Branched-chain amino acids: Enzyme and substrate regulation. The Journal of Nutrition, 136, 207-211.

Castillo, S., Halligan, S., Gatlin III, D.M. (2015). Growth responses of Juvenile red drum *Sciaenops ocellatus* to dietary phenylalanine and tyrosine can be used to calculate the total aromatic amino acid requirement. The Journal of Nutrition, 145, 2341-2346.

Chance, R., Mertz, E., Halver, J. (1964). Nutrition of salmonoid fishes: XII. Isoleucine, leucine, valine and phenylalanine requirements of Chinook salmon and interrelations between isoleucine and leucine for growth. The Journal of Nutrition, 83, 177-185.

Choo, P., Smith, T., Cho, C., Ferguson, H. (1991). Dietary excesses of leucine influence growth and body composition of rainbow trout. The Journal of Nutrition, 121, 1932-1939.

Cowey, C.B. (1994). Amino acid requirements of fish: a critical appraisal of present values. Aquaculture, 124, 1-11.

Craig, S., Gatlin III, D.M. (1992). Dietary lysine requirement of juvenile red drum *Sciaenops ocellatus*. Journal of the World Aquaculture Society, 23, 133-137.

De Silva, S.S., Gunasekera, R.M., Gooley, G. (2000). Digestibility and amino acid availability of three protein-rich ingredient incorporated diets by Murray cod *Maccullochella peelii peelii* (Mitchell) and the Australian shortfin eel *Anguilla australis* Richardson. Aquaculture Research, 31, 195–205.

Encarnação, P., de Lange, C., Rodehutscord, M., Hoehler, D., Bureau, W., Bureau, D.P. (2004). Diet digestible energy content affects lysine utilization, but not dietary lysine requirements of rainbow trout (*Oncorhynchus mykiss*) for maximum growth. Aquaculture, 235, 569-586.

FAO (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. FAO, Rome.

Folch, J., Lees, M., Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. The Journal of Biological Chemistry, 226, 497-507.

Gan, L., Zhou, L.-L., Li, X.-X., Yue, Y.-R. (2015). Dietary leucine requirement of Juvenile Nile tilapia, *Oreochromis niloticus*. Aquaculture Nutrition, 22, 1040-1046.

Gatlin III, D.M. (2002). Red Drum, *Sciaenops ocellatus,* in: Webster, C., Lim, C. (Eds.), Nutrient Requirements and Feeding of Finfish for Aquaculture. CABI, New York, USA, pp. 147-171.

Giri, S.S., Sen., S.S., Chi, C., Kim, H.J., Yun, S., Park, S.C., Sukumaran, V. (2015). Effect of dietary leucine on the growth parameters and expression of antioxidant, immune, and inflammatory genes in the head kidney of *Labeo rohita* fingerlings. Veterinary Immunology and Immunopathology, 167, 36-43.

Guillet, C., Delcourt, I., Rance, M., Giraudet, C., Walrand, S., Bedu, M., Duche, P., Boirie, Y. (2009). Changes in basal and insulin and amino acid response of whole body and skeletal muscle proteins in obese men. The Journal of Clinical Endocrinology and Metabolism, 94, 3044-3050.

Han, Y., Han, R., Koshio, S, Ishikawa, M., Yokoyama, S., Gao, J. (2014). Interactive effects of dietary valine and leucine on two sizes of Japanese flounder *Paralichthys olivaceus*. Aquaculture, 432, 130-138.

Huang, Z., Tan, X.-h., Zhou, C.-p., Yang, Y.-k., Qi, C.-I., Zhao, S.-y., Lin, H.-z. (2017). Effect of dietary valine levels on the growth performance, feed utilization and immune function of juvenile golden pompano, *Trachinotus ovatus*. Aquaculture Nutrition, early view.

Hughes, S.G., Rumsey, G.L., Nesheim, M.C. (1984). Effects of dietary excesses of branched-chain amino acids on the metabolism and tissue composition of lake trout (*Salvelinus namaycush*). Comparative Biochemistry and Physiology Part A: Physiology, 78, 413-418.

Kaushik, S.J., Luquet, P. (1979). Influence of dietary amino acid patterns on the free amino acid contents of blood and muscle of rainbow trout *Salmo gairdneri*. Comparative Biochemistry and Physiology B, 64, 175-180.

Kaushik, S.J., Seiliez, I. (2010). Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. Aquaculture Research, 41, 322-332.

Kawanago, M., Takemura, S., Ishizuka, R., Kousaka, T., Shioya, I. (2014). Leucine Affects Growth and Hepatic Growth-Related Factor Gene Expression in Japanese Amberjack *Seriola quinqueradiata*. North American Journal of Aquaculture, 76, 415-422.

Kawanago, M., Takemura, S., Ishizuka, R., Shioya, I. (2015). Dietary branched-chain amino acid supplementation affects growth and hepatic insulin-like growth factor gene expression in yellowtail, *Seriola quinqueradiata*. Aquaculture Nutrition, 21, 63-72.

Khan, M.A., Abidi, S.F. (2007). Dietary isoleucine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton). Aquaculture Nutrition, 13, 424-430.

Kim, K.I., Kayes, T.B., Amundson, C.L. (1992). Requirements for lysine and arginine by rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 106, 333-344.

Kim, S., Buel, G., Blenis, J. (2013). Nutrient regulation of the mTOR complex I signaling pathway. Molecular Cell, 35, 463-473.

Lansard, M., Panserat, S., Plagnes-Juan, E., Dias, K., Seiliez, I., Skiba-Cassy, S. (2011). L-Leucine, L-Methionine, and L-Lysine Are Involved in the Regulation of Intermediary Metabolism-Related Gene Expression in Rainbow Trout Hepatocytes. The Journal of Nutrition, 141, 75-80.

Mambrini, M., Kaushik, S.J. (1995). Indispensable amino acid requirements of fish: Correspondence between quantitative data and amino acid profiles of tissue proteins. Journal of Applied Ichthyology, 11, 240-247.

Matsumoto, T., Nakamura, K., Matsumoto, H., Sakai, R., Kuwahara, T., Kadota, Y., Kitaura, Y., Sato, J., Shimomura, Y. (2014). Bolus ingestion of individual branched-chain amino acids alters plasma amino acid profiles in young healthy men. SpringerPlus, 3, 35.

May, R.C., Piepenbrock, N., Kelly, R.A., Mitch, W.E. (1991). Leucine-Induced Amino Acid Antagonism in Rats: Muscle Valine Metabolism and Growth Impairment. The Journal of Nutrition, 121, 293-301.

McGoogan, B.B., Gatlin III, D.M. (1998). Effects of replacing fish meal with soybean meal in diets for red drum *Sciaenops ocellatus* and potential for palatability enhancement. Journal of the World Aquaculture Society, 28, 374-385.

Moon, H.-Y., Gatlin III, D.M. (1991). Total sulfur amino acid requirement of juvenile red drum, *Sciaenops ocellatus*. Aquaculture, 95, 97-106.

Newgard, C.B., An, J., Bain, J.R., Muehlbauer, M.J., Stevens, R.D., Lien, L.F., Haqq, A.M., Shah, S.H., Arlotto, M., Slentz, C.A., Rochon, J., Gallup, D., Ilkayeva, O., Wenner, B.R., Yancy, W.S., Eisenson, H., Musante, G., Surwit, R., Millington, D.S., Butler, M.D., Svetkey, L.P. (2009). A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metabolism, 9, 565-566.

Nose, T. (1979). Summary report on the requirements of essential amino acids for carp. In: Halver, J.E., Tiews, K. (Eds.) Finfish Nutrition and Fishfeed Technology, vol 1. Heinemann, GmbH, Berlin, Germany, pp. 145–156.

NRC (2011). Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washington, D.C. 376 pp.

Paxton, R., Harris, R.A. (1984). Regulation of branched-chain α-keto acid dehydrogenase kinase. Archives of Biochemistry and Biophysics, 231, 48-57.

Pewitt, E., Castillo, S., Velásquez, A., Gatlin III, D.M. (2017). The dietary tryptophan requirement of juvenile red drum, *Sciaenops ocellatus.* Aquaculture, 469, 112-116.

Poston, H.A., Riis, R.C., Rumsey, G.L., Ketola, H.G. (1977). The effect of supplemental dietary amino acids, minerals and vitamins on salmonids fed cataractogenic diets. The Cornell Veterinarian, 167, 472–509.

Rahimnejad, S., Lee, K.-J. (2013). Dietary valine requirement of juvenile red sea bream *Pagrus major*. Aquaculture, 416-417, 212-218.

Ren, M.-C., Habte-Tsion, H.-M., Liu, B., Miao, L., Ge, X., Xie, J., Liang, H., Zhou, Q., Pan, L. (2015a). Dietary leucine level affects growth performance, whole body composition, plasma parameters and relative expression of TOR and TNF-α in juvenile snout bream, *Megalobrama amblycephala*. Aquaculture, 448, 162-168.

Ren, M.-C., Habte-Tsion, H.-M., Liu, B., Zhou, Q.-L., Xie, J., Ge, X.-P., Liang, H.-L., Zhao, Z.-X. (2015b). Dietary valine requirement of juvenile blunt snout bream (*Megalobrama amblycephala* Yih, 1955). Journal of Applied Ichthyology, 31, 1086-1092.

Robbins, K.R., Saxton, A.M., Southern, L.L. (2006). Estimation of nutrient requirements using broken-line regression analysis. Journal of Animal Sciences, 84, 155-165.

Robinson, E.H., Poe, W.E., Wilson, R.P. (1984). Effects of feeding diets containing an imbalance of branched-chain amino acids on fingerling channel catfish. Aquaculture, 37, 51-62.

Rodehutscord, M., Becker, A., Pack, M., Pfeffer, E. (1997). Response of rainbow trout (*Oncorhynchus mykiss*) to supplements of individual essential amino acids in a semipurified diet, including an estimate of the maintenance requirement for essential amino acids. The Journal of Nutrition, 127, 1166-1175.

Santiago, C.B., Lovell, R.T. (1988). Amino acid requirements for growth of Nile tilapia. The Journal of Nutrition, 118, 1540–1546.

Serrano, J., Nematipour, G., Gatlin, D. (1992). Dietary protein requirement of the red drum (*Sciaenops ocellatus*) and relative use of dietary carbohydrate and lipid. Aquaculture, 101, 283-291.

Sheriff, D.S., Younis, M.Y.G., Elshaari, F.A., Mohamed, N.A., El-Kuwaila, H.I.A., Abdalla, S.A.S., Elfaghi, R. (2014). A perspective on interaction between lipid and branched chain amino acids (BCAA) in developing insulin resistance. Medicine Journal, 1, 8-12.

Simmons, L., Moccia, R.D., Bureau, D.P., Sivak, J.G., Herbert, K. (1999). Dietary methionine requirement of juvenile Arctic charr *Salvelinus alpinus* (L.). Aquaculture Nutrition, 5, 93–100.

Swendseid, M.E., Villalobos, J., Figueroa, W.S., Drenick, E.J. (1965). The Effects of Test Doses of Leucine, Isoleucine or Valine on Plasma Amino Acid Levels: The Unique Effect of Leucine. The American Journal of Clinical Nutrition, 17, 317-321.

Tan, X., Lin, H., Huang, Z., Zhou, C., Wang, A., Qi, C., Zhao, S. (2016). Effects of dietary leucine on growth performance, feed utilization, non-specific immune responses and gut morphology of juvenile golden pompano *Trachinotus ovatus*. Aquaculture, 465, 100-107.

Waagbø, R., Tröße, C., Koppe, W., Fontanillas, R., Breck, O. (2010). Dietary histidine supplementation prevents cataract development in adult Atlantic salmon, *Salmo salar* L., in seawater. British Journal of Nutrition, 104, 1460-1470.

Walton, M.J., Coloso, R.M., Cowey, C.B., Adron, J.W., Knox, D. (1984a). The effects of dietary tryptophan levels on growth and metabolism of rainbow trout (*Salmo gairdneri*). British Journal of Nutrition, 51, 279–287.

Walton, M.J., Cowey, C.B., Coloso, RM., Adron, J.W. (1984b). Dietary requirements of rainbow trout for tryptophan, lysine and arginine determined by growth and biochemical measurements. Fish Physiology and Biochemistry, 2, 161-169.

Wang, L., Han, Y., Jiang, Z., Sun, M., Si, B., Chen, F., Bao, N. (2017). Interactive effects of dietary leucine and isoleucine on growth, blood parameters, and amino acid profile of Japanese flounder *Paralichthys olivaceus*. Fish Physiology and Biochemistry, 43, 1265-1278.

Williams, K., Barlow, C., Rodgers, L. (2001). Efficacy of crystalline and protein-bound amino acids for amino acid enrichment of diets for barramundi/Asian seabass (*Lates calcarifer* Bloch). Aquaculture Research, 32, 415-429.

Wilson, R.P., Poe, W.E., Robinson, E.H. (1980). Leucine, isoleucine, valine and histidine requirements of fingerling channel catfish. The Journal of Nutrition, 110, 627-633.

Yamamoto, T., Shima, T., Furuita, H. (2004). Antagonistic effects of branched-chain amino acids induced by excess protein-bound leucine in diets of rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 232, 539-550.

Zehra, S., Khan, M.A. (2014). Dietary Valine Requirement of Fingerling *Catla catla*. Journal of Applied Aquaculture, 26, 232-251.

Zehra, S., Khan, M.A. (2015). Dietary leucine requirement of fingerling *Catla catla* (Hamilton) based on growth, feed conversion ratio, RNA/DNA ratio, leucine gain, blood indices and carcass composition. Aquaculture International, 23, 577-595.

Zhang, S., Zeng, X., Ren, M., Mao, X., Qiao, S. (2017). Novel metabolic and physiological functions of branched chain amino acids: a review. Journal of Animal Science and Biotechnology, 8, 1-12