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Growth Performance of Adult Florida Pompano, *Trachinotus carolinus*, Fed Semi-purified Diets with Graded Levels of Methionine

Christina M. Belfranin

University of Miami, cbelfranin@gmail.com

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UNIVERSITY OF MIAMI

GROWTH PERFORMANCE OF ADULT FLORIDA POMPANO, *Trachinotus carolinus*, FED SEMI-PURIFIED DIETS WITH GRADED LEVELS OF METHIONINE

By

Christina M. Belfranin

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

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August 2016

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Christina M. Belfranin

Approved:

Daniel D. Benetti, Ph.D.
Professor, Marine Affairs and Policy
Director of Aquaculture

Jorge A. Suarez, Ph.D.
Research Scientist Associate,
Marine Affairs and Policy

Delbert M. Gatlin III, Ph.D.
Regents Professor and Associate
Head, Department of Wildlife
and Fisheries Sciences
College Station, Texas

Guillermo Prado, Ph.D.
Dean of the Graduate School

BELFRANIN, CHRISTINA M.

(M.S., Marine Affairs and Policy)
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Growth Performance of Adult Florida
Pompano, *Trachinotus carolinus*, Fed
Semi-purified Diets with Graded Levels
of Methionine

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Replacement of fishmeal with soy-based protein alternatives has produced the need to determine nutritional requirements of aquaculture species to appropriately formulate and supplement aquafeeds. Compared to fishmeal, soybean meal is deficient in methionine, an essential amino acid. Thus, the methionine requirement of a high-value and soy-tolerant species, the Florida pompano (*Trachinotus carolinus*) at a near harvestable size (>200 g) was evaluated. Six semi-purified, isonitrogenous and isocaloric, experimental diets were formulated based on the whole-body amino acid (AA) profile of the Florida pompano with incremental inclusions of methionine (0.5, 0.7, 0.9, 1.1, 1.3, and 1.5 g 100 g diet⁻¹). Three tanks were randomly assigned per diet and stocked with 11 fish per tank averaging a total of 2290.3 ± 13.86 g initial biomass. Fish were fed twice daily at 900 h and 1500 h to satiation for 58 days. Pompano from all treatments, including the lowest and highest inclusions of methionine exhibited normal behavior, appearance, and weight gain throughout the duration of the experiment. There were no discernable trends (neither increasing nor decreasing) among growth parameters across all methionine inclusion levels. No significant differences ($p > 0.05$) were observed among

treatments for mean daily intake (MDI), weight gain ratio (WGR), specific growth rate (SGR), energy intake (EI), protein intake (PI), feed efficiency (FE), and hepatosomatic index (HSI). Significant differences were apparent ($p < 0.05$) for average weight gain (AWG), average daily gain (ADG), protein efficiency ratio (PER), and feed conversion ratio (FCR) with substandard values for fish fed Diet 4 ($1.1 \text{ g } 100 \text{ g diet}^{-1}$) compared to the other diets. These results suggest that near-harvestable size Florida pompano perform well with respect to growth at the lowest methionine inclusion of $0.5 \text{ g } / 100 \text{ g diet}$. Further research is required to confirm these findings.

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Chapter 1: Introduction

As the aquaculture industry continues to expand, discovery of novel protein sources for aquaculture feeds are necessary and in demand. Until recently, fishmeal (FM) has been the primary source of protein for feeds used in the production of carnivorous marine finfish and crustaceans (National Research Council, 2011). FM and fish oil (FO) provide farmed seafood with the omega-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) that consumers seek because of their health benefits (National Research Council, 2011). Although FM and FO deliver a nearly ideal nutritional profile (National Research Council, 2011), they are becoming more limited in supply due to the worldwide growth of commercial aquaculture which is projected to continue in the future to meet the world's escalating demand for seafood (FAO, 2014). High costs and ecological consequences from sourcing FM have driven feed manufacturers to utilize alternative proteins that do not compromise growth or feed efficiency (Naylor et al., 2009). Additionally, the present consumer is not only concerned with the sustainability of farmed seafood, but with the possible contaminants found in aquafeeds. These contaminants can range from heavy metals and persistent organic pollutants to mycotoxins and excess minerals (Tacon and Metian, 2008). Bioaccumulation of contaminants, specifically persistent organic pollutants and heavy metals, in wild fish has been a major issue regarding the quality of FM and FO (National Research Council, 2011). For these reasons, the development of aquaculture will rely on innovative ingredients that are nutritionally comparable to FM and FO, yet ecologically sourced. (National Research Council, 2011).

The following review will focus on the investigation of FM replacement as it relates to farmed marine finfish. Potential, non-fish protein alternatives include, but are not limited to: terrestrial plant-based proteins such as soybeans, peanuts, wheat, and corn; rendered terrestrial animal products such as poultry by-product meal, feather meal, meat and bone meal; and single-cell proteins (National Research Council, 2011; Naylor et al., 2009). These ingredients vary in price, accessibility, ease of manufacture, and nutrition. For example, rendered terrestrial animal products like meat and bone meal are cost-effective and nutritionally similar to FM in terms of their amino acid profiles, but there is concern over the risk of disease dispersal such as transmissible spongiform encephalopathies (National Research Council, 2011; Naylor et al., 2009). Single-cell proteins such as various species of microalgae, are an excellent source of protein and lipid, but currently have exceedingly high production costs (Naylor et al., 2009). Lastly, several plant-based proteins like soybean meal, peanut meal, and wheat gluten are readily available and reasonably cost effective, but need nutritional supplementation due to lower levels of amino acids compared to FM (National Research Council, 2011). There is a wide range of available protein alternatives; however, the successful partial or total replacement of FM relies on the alternatives' digestibility, palatability, and nutritional bioavailability, which are species dependent.

Fish in general require high levels of protein in their diets, up to 2 to 4 times more than other vertebrates, and carnivorous marine fish, in particular, require even higher levels than freshwater fish (Wilson, 2003). Over several decades ago, fish feeds for carnivorous species generally contained 20 – 60% FM (Watanabe, 2002) to meet high protein requirements. As the demand for FM has increased with the expansion of

aquaculture, these feed ingredients have become very expensive. Therefore, utilizing less expensive and more sustainable protein sources would be very beneficial to feed manufacturers and aquaculture as a whole. Of the alternatives previously mentioned, soybean meal is of particular interest for its availability, economical price, and high protein content (National Research Council, 2011). According to Akiyama (1988), soybean meal is palatable to most fish species and provides a more complete amino acid profile when compared to other plant-based alternatives. Watanabe (2002) described defatted soybean meal as qualitatively and quantitatively accepted by most farmed fish species. There have been several FM replacement studies utilizing different soybean products on various species, which have yielded promising results. One such study demonstrated that juvenile cobia (*Rachycentron canadum*) can exhibit satisfactory growth with up to 75% FM replacement utilizing a combination of soybean meal and soy protein concentrate (SPC) (Salze et al., 2010). Similarly, juvenile Florida pompano (*Trachinotus carolinus*) displayed no difference in weight gain or feed efficiency when fed a diet of 80% FM replacement with solvent- extracted soybean meal compared to the 100% FM control diet (Riche and Williams, 2011). Shiu et al. (2015) found no significant differences in growth performance or feed efficiency of orange-spotted grouper, *Epinephelus coioides*, fed control diets and the 30% FM replacement with fermented soybean meal diet.

Although there has been success in the partial replacement of FM with soybean-based proteins, there are limitations associated with plant meals that need to be considered when formulating feeds. A high content of carbohydrates is of concern since carnivorous marine fish cannot readily digest certain complex sugars, primarily non-

starch polysaccharides (Gatlin III et al., 2007; National Research Council, 2011). Unlike FM, plant-based proteins may contain several anti-nutritional factors. Soybean in particular, contains trypsin inhibitors and phytic acid, among others (Hardy and Barrows, 2003). The trypsin inhibitors can be inactivated by heat (Hardy and Barrows, 2003), while the enzyme phytase can be added to feeds to ameliorate the effects of phytic acid (Gatlin III et al., 2007). Another issue arises through inadequate amino acid (AA) profiles, commonly yielding insufficient levels of the essential amino acids (EAA), the most limiting being lysine, methionine, and threonine in soy-based aquafeeds (Gatlin III et al., 2007). Synthetic crystalline amino acids can be supplemented in feed formulations to compensate for limitations and balance amino acid compositions (National Research Council, 2011). The various species of farmed marine fish have distinct dietary requirements, which need to be accurately estimated to develop appropriate feed formulations especially when substituting FM (National Research Council, 2011).

A key area of fish nutrition research is the optimization of dietary requirements for AAs (Li et al., 2008). In contrast to high quality FM, which contains a reasonable balance of all 10 EAA for fish (Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, and Valine) at sufficient levels, soy-based proteins contain these EAAs, but some are at inadequate levels (National Research Council, 2011). Therefore, determining species-specific AA requirements are necessary for the precise formulation of soy-based feeds to meet metabolic requirements.

Developing feeds containing appropriate AA profiles will be beneficial to farmers by reducing waste and making feeds more cost-effective. Because feeds present one of the

highest costs in aquaculture operations, fine-tuning species-specific needs with reliable estimates of dietary requirements would be advantageous.

A species of renewed interest that tolerates soy-based diets and has a high market value is the Florida pompano. Several characteristics deem pompano as a viable aquaculture candidate such as its euryhaline physiology (Allen and Avault, 1970), its hardiness, and its ability to be spawned and subsequently reared from egg to market in captivity (McMaster, 1988; Weirich and Riley, 2007). Being a carnivorous, marine fish, pompano have a protein requirement of approximately 45% by weight (Lazo et al., 1998; Riche, 2009; Taynor, 2013). However, there is limited available research on other nutritional requirements of this particular species and no nutritionally balanced, practical diet exists for it (Riche and Williams, 2010). This presents major obstacles for the expansion and commercialization of pompano aquaculture. By investigating the nutritional requirements of pompano, environmentally sustainable and economical diets may be developed to further the establishment of pompano aquaculture.

The most limiting AA in soy-based aquafeeds are the sulfur amino acids (SAA), methionine (Met) and cystine (Cys) (Goff and Gatlin III, 2004; Keembiyehetty and Gatlin III, 1997). Met is an EAA that fish are incapable of synthesizing and it must be provided in a diet (National Research Council, 2011). Conversely, Cys is a nonessential AA (NEAA) as a result of its synthesis from Met. These SAA play important roles in protein synthesis and structure (National Research Council, 2011). Therefore, it is a priority to quantify the total sulfur amino acid (TSAA) requirement of Florida pompano so feeds for this species can be appropriately supplemented. Through several different metabolic reactions, Met is responsible for the production of S-adenosylmethionine

(SAM), cystine, glutathione, taurine, phosphatidylcholine, and other phospholipids (Brosnan and Brosnan, 2006). Other products indirectly synthesized by Met through SAM include creatine, epinephrine, melatonin, spermine, and spermidine (Brosnan and Brosnan, 2006). These substances play an important role in several physiological processes in fish. For instance, spermine induces intestinal maturation in larval sea bass, *Dicentrarchus labrax* (Li et al., 2008; Péres et al., 1997); creatine has been shown to function as an antioxidant and provide cellular, high energy storage in Arctic charr, *Salvelinus etabol* (Bystriansky et al., 2007; Li et al., 2008); and glutathione plays a key role in antioxidant defense, nutrient metabolism, and cellular events for all animals (Li et al., 2008; Wu et al., 2004).

When diets are limited in SAA, fish can experience a variety of consequences including reduced weight gain (National Research Council, 2011), impaired feed intake (Tulli et al., 2010), and reduced survival (Savolainen and Gatlin III, 2010). Diets limited in Met have shown to cause bilateral cataracts in salmonid species (Poston et al., 1977) and in hybrid striped bass, *Morone chrysops* × *M. saxatilis* (Keembiyehetty and Gatlin III, 1993). Research has shown that supplementing soy-based aquafeeds with Met improves the growth performance of several species (Ai and Xie, 2005; Alam et al., 2012; Figueiredo-Silva et al., 2015). Studies on rainbow trout, *Oncorhynchus mykiss* (Cowey et al., 1992; Rumsey et al., 1983; Walton et al., 1982) among several other fish species have indicated that the Met requirement decreases when dietary Cys is provided in the diet (Abidi and Khan, 2011; Goff and Gatlin III, 2004; Moon and Gatlin III, 1991). This Met sparing effect by Cys complicates determining a dietary Met requirement, thus the TSAA value is estimated in its place (National Research Council, 2011). The TSAA requirement

can be met by the appropriate content of Met, or by the proper ratio of Met:Cys (Moon and Gatlin III, 1991).

The TSAA requirement for pompano, particularly juvenile to adult sized pompano, is unknown and thus far, no AA requirement studies have been conducted for the species. However, the optimal dietary Met requirement for a related species, golden pompano (*Trachinotus ovatus*), has been quantified for juveniles (initial weight, 12.40 ± 0.02 g and final weight, 51.81 – 81.58 g) to be between 1.06 and 1.27 % of diet, corresponding to 2.46 – 2.95 % of dietary protein (Niu et al., 2013). These requirement estimates conform to previous studies summarized by Niu et al. (2013) on juvenile Japanese flounder (1.49 % of diet, and 3.1 % of dietary protein; Alam et al., 2001), juvenile orange-spotted grouper (1.31 % of diet, and 2.73 % of dietary protein; Luo et al., 2005), large yellow croaker (1.44 % of diet, and 3.34 % of dietary protein; Mai et al., 2006), juvenile cobia (1.19 % of diet, and 2.64 % of dietary protein; Zhou et al., 2006), and juvenile rockfish (1.37 % of protein, and 2.80 % of dietary protein; Yan et al., 2007). Although these results provide a reasonable foundation for investigating the Florida pompano's TSAA requirement, they are values for a smaller size class of fish. Smaller organisms require more protein than larger animals (Lee et al., 2015; Wilson, 2003), thus fish in later life stages (sub-adult, adult) or at a near-harvestable size, will require less protein and may therefore require different inclusions of AA in their diets. This necessitates research to determine requirements in large fish (> 200 g), which demand higher volumes of feed than earlier life stages, resulting in higher costs for producers. If more sustainable soy-based diets can be formulated with more precise estimates of AA requirements of large fish, then the expense of supplementing feeds with limiting EAAs

may be reduced, thus improving the economic efficiency of producing species such as pompano.

Pompano can readily digest different soy proteins such as soybean meal, soy protein isolate, and soy protein concentrate at various inclusion rates in practical diets with partial to total FM replacement (Quintero et al., 2012; Riche and Williams, 2011, 2010; Taynor, 2013). However, the majority of research completed on pompano has been concerned with larval (Cassiano et al., 2011; Cavalin and Weirich, 2009) or early juvenile life stages (Lazo et al., 1998; Riche, 2009; Tatum, 1972; Williams et al., 1985) up to 112 grams in size (González-Félix et al., 2010). Few studies have utilized sub-adult or adult sized pompano from 200 – 500 g, which include an evaluation of gross protein/gross energy ratio (Taynor, 2013), effects of feeding strategies on growth in closed recirculating systems (Groat, 2002), and effect of density on growth in low-salinity recirculating aquaculture systems (Weirich et al., 2009). The high cost of manufacturing experimental diets and the large volume necessary to grow fish out to market size (approximately 500 g for pompano) may explain the lack in determining AA requirements of large fish. Nevertheless, such experiments on large fish are pertinent to the advancement of pompano aquaculture.

The first qualitative AA requirement studies for fish were completed by Halver et al. (1957) on chinook salmon, *Oncorhynchus tshawytscha*, in 1957 (Wilson, 2003). Test diets used for that work were developed based on the AA profile of whole chicken egg protein by Halver (1957), which outperformed AA profiles of chinook salmon egg protein and chinook yolk sac fry protein (Wilson, 2003). The 10 EAAs Halver et al. (1957) discovered for chinook salmon have been found to be essential for all species

studied since (Wilson, 2003). Thereafter, quantitative analysis of individual AA requirements have been determined for several species by feeding experimental diets containing graded levels of the AA of interest and following the AA profile of whole chicken egg protein (Wilson, 2003). Test diets for these experiments can be purified, meaning the source of protein is all crystalline AA or a combination of AA with gelatin or casein; semipurified, being an imbalanced protein, limited in certain AA combined with crystalline AA; or practical-type, using an intact protein such as FM in combination with crystalline AA (Wilson, 2003). However, for the past several decades fish nutritionists have adopted the ideal protein concept in which the whole-body amino acid profile of an organism is directly correlated to the dietary AA requirements of that organism, and have developed test diets based on whole-body AA profiles (Wilson, 2003). Several studies presented by Furuya et al. (2004) have reported high correlations between whole-body tissue AA patterns and AA requirement patterns for channel catfish, *Ictalurus punctatus* (Wilson and Poe, 1985), silver perch, *Bidyanus bidyanus* (Ngamsnae et al., 1999), and several other species (Akiyama et al., 1997). A dose-response study can be developed using one of the test diets previously mentioned to estimate dietary AA requirements following whole-tissue AA profiles.

The ideal protein concept assumes that the efficient utilization of the first limiting EAA decreases as the inclusion of digestible protein increases (National Research Council, 2011). The first limiting EAA is often lysine for several feedstuffs, but can also be methionine such as in soy-based diets. As the digestible protein/digestible energy ratio (DP/DE) increases in feeds, more AAs are catabolized for energy and it is assumed that the first limiting EAA would not be spared from at the expense of other non-essential

amino acids or non-limiting EAAs (National Research Council, 2011). Therefore, the expression of the other 9 EAAs as a ratio to the first limiting EAA produces a need to balance the AA profile for proper metabolism and efficient growth. By attaining the first limiting EAA requirement at different sizes and under various conditions through traditional dose response studies, the values of the remaining EAAs can be estimated (National Research Council, 2011). Using lysine as an example, this is done by attaining the whole body AA pattern of the species and setting lysine to an arbitrary value of 100 and subsequently calculating the ratios of the other 9 EAAs based on that information. The lysine requirement value allows for the proper balance of each of the remaining EAAs making them all equally limiting. AA requirements made based on the ideal protein concept may be applied to low-protein diets that will yield the optimum, efficient uptake of protein with minimal nitrogenous waste (National Research Council, 2011).

Because there are no AA requirement studies performed on Florida pompano to date, the ideal protein concept cannot be applied, but a traditional dose response study would be the first step to attain the TSAA requirement of this species. After the proper TSAA requirement is quantified, it can be applied to the ideal protein concept. The remaining EAAs could then be estimated using the value of methionine and the whole-body AA pattern of the Florida pompano.

Generally, a growth response curve based on weight gain is used to estimate the optimal AA requirement in a dose-response trial. The growth curve usually increases linearly as the inclusion of the AA of interest increases until reaching a point of inflection where the growth curve plateaus, indicating an optimal level of the particular AA (Wilson, 2003). Different statistical methods have been used to estimate the precise AA

requirement based on the growth curve breakpoint of a dose-response trial (Wilson, 2003). A few of these methods include regression analyses, such as the continuous broken-line method developed by Robbins et al. (1979) and quadratic regression analysis. The broken-line method has been commonly used to estimate requirement values for several species (Abidi and Khan, 2011; Luo et al., 2005; Moon and Gatlin III, 1991; Niu et al., 2013).

The methods discussed above will be used to investigate the TSAA requirement of sub-adult Florida pompano. The main objective of the investigation is to develop economical, yet environmentally sustainable, soy-based diets for Florida pompano. The outcome of the study will aid in the continuous expansion of the aquaculture industry, especially the development of pompano aquaculture.

Chapter 2: Materials and Methods

2.1 Experimental Diets

Six semi-purified, isonitrogenous and isocaloric, experimental diets were formulated with incremental inclusions of Met (Table 1) ranging from 0.5 % by weight in Diet 1 to 1.5 % by weight in Diet 6. The six formulations all shared equal inclusion levels of four practical ingredients: fishmeal at 12.5 %, regular soybean meal at 7.5 %, soy protein concentrate at 7.5 %, and wheat flour at 12 %. These four ingredients provided approximately 21.5 % protein to the diets. The total inclusion of crystalline AAs (Table 2), based on the whole-body amino acid composition of the pompano, was approximately 19.2 %. This resulted in diets that contained approximately 40.7 % crude protein, which is less than the optimum crude protein inclusion of 45 % for Florida pompano (Lazo et al., 1998; Riche, 2009; Taynor, 2013). This method was employed to guarantee the efficient uptake of AA in the process of protein synthesis to attain an accurate amino acid requirement estimate. The different crystalline AAs were at equal inclusion levels for all six diets except for Met and the Aspartic/Glutamic acid mix. The inclusion of crystalline Met was increased in approximate increments of 0.20 % starting with 0.0 % in Diet 1 up to 1.04 % in Diet 6. As Met was increased, the Aspartic/Glutamic acid mix was decreased to assure isonitrogenous diets.

Table 1. Composition and proximate analysis of six experimental diets with graded levels of methionine.

Diets (Met g · 100 g diet ⁻¹)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fish meal ¹	12.5	12.5	12.5	12.5	12.5	12.5
Soybean meal, solvent- extracted ²	7.5	7.5	7.5	7.5	7.5	7.5
Soy protein concentrate ³	7.5	7.5	7.5	7.5	7.5	7.5
Wheat flour ⁴	12	12	12	12	12	12
Amino acid mix ⁵	18.17	18.17	18.17	18.17	18.17	18.17
Fish oil ¹	13.73	13.73	13.73	13.73	13.73	13.73
Choline chloride ²	0.23	0.23	0.23	0.23	0.23	0.23
Lecithin ⁶	1	1	1	1	1	1
Ethoxyquin ⁷	0.03	0.03	0.03	0.03	0.03	0.03
Vitamin/Mineral premix ⁸	1	1	1	1	1	1
Celufil ⁹	25.31	25.31	25.31	25.31	25.31	25.31
Crystalline DL- methionine ²	0.00	0.22	0.42	0.62	0.82	1.04
Glutamic Acid ¹⁰	0.52	0.41	0.31	0.21	0.11	0.00
Aspartic Acid ¹⁰	0.52	0.41	0.31	0.21	0.11	0.00
Proximate analysis, on dry-weight basis (g · 100 g diet ⁻¹)						
Crude protein	42.07	42.15	42.81	41.83	41.61	40.28
Crude lipid	16.17	15.72	15.07	15.84	15.61	15.95
Moisture	5.61	4.77	4.28	4.96	5.84	5.32
Ash	2.20	2.04	2.04	2.02	2.06	1.96
Methionine	0.52	0.71	0.92	1.11	1.29	1.56
Energy (kcal · g ⁻¹)	5.36	5.39	5.33	5.41	5.38	5.40

¹ Bio-Oregon Proteins

² ADM Company, Decatur, Illinois

³ Solae Company, St. Louis, Missouri

⁴ Manildra Milling, Fairway, Kansas

⁵ Amino acid mix (%): Arginine, 1.49 %; Histidine 0.51%; Isoleucine, 1.03%; Leucine, 1.62 %; Lysine 2.01 %; Phenylalanine, 0.90 %; Threonine, 0.98 %; Valine, 1.24 %; Tryptophan, 0.43 %; Alanine, 2.24 %; Glycine, 3.20 %; Proline, 1.23 %; Serine, 0.66 %; Taurine, 0.46%; Tyrosine, 0.17 %

⁶ American Lecithin Company, Oxford, Connecticut

⁷ Sigma Alderich, St. Louis, Missouri

⁸ Formulation by Dr. Jorge Suarez; mix manufactured by Ridley Feed Ingredients, Mendota, Illinois

⁹ MP Biomedicals, Santa Ana, California

¹⁰ Affymetrix, Santa Clara, California

Table 2. Crystalline amino acid inclusion in experimental diet formulations.

Amino acids (g · 100 g diet ⁻¹)	Diets					
	D1	D2	D3	D4	D5	D6
EAA						
Arginine ¹	1.49	1.49	1.49	1.49	1.49	1.49
Histidine ¹	0.51	0.51	0.51	0.51	0.51	0.51
Isoleucine ¹	1.03	1.03	1.03	1.03	1.03	1.03
Leucine ¹	1.62	1.62	1.62	1.62	1.62	1.62
Lysine ²	2.01	2.01	2.01	2.01	2.01	2.01
DL-Methionine ^{2†}	0.00	0.22	0.42	0.62	0.82	1.04
Phenylalanine ¹	0.90	0.90	0.90	0.90	0.90	0.90
Threonine ²	0.98	0.98	0.98	0.98	0.98	0.98
Valine ¹	1.24	1.24	1.24	1.24	1.24	1.24
Tryptophan ¹	0.43	0.43	0.43	0.43	0.43	0.43
NEAA						
Alanine ¹	2.24	2.24	2.24	2.24	2.24	2.24
Aspartic Acid ^{1†}	0.52	0.41	0.31	0.21	0.11	0.00
Cystine	0.00	0.00	0.00	0.00	0.00	0.00
Glutamic Acid ^{1†}	0.52	0.41	0.31	0.21	0.11	0.00
Glycine ¹	3.20	3.20	3.20	3.20	3.20	3.20
Proline ³	1.23	1.23	1.23	1.23	1.23	1.23
Serine ¹	0.66	0.66	0.66	0.66	0.66	0.66
Taurine ⁴	0.46	0.46	0.46	0.46	0.46	0.46
Tyrosine ¹	0.17	0.17	0.17	0.17	0.17	0.17

† Values for crystalline amino acids were adjusted for increasing methionine levels

¹ Affymetrix, Santa Clara, California

² ADM Company, Decatur, Illinois

³ Sigma Alderich, St. Louis, Missouri

⁴ Nusci Institute and Corporation, Walnut, California

Appropriate quantities of the crystalline AAs were supplemented by referencing the AA composition of the Florida pompano whole-body tissue. Prior to the feeding trial, five pompano were randomly selected, frozen, ground, and homogenized before being lyophilized (Labconco Kansas City, MO, USA). The lyophilized pompano tissue was then sent to Texas A&M University for AA composition analyses. Basal quantities of the various AA were provided by the four practical ingredients previously mentioned, and then were supplemented by the addition of crystalline AA from Sigma-Aldrich (St. Louis, MO, USA) and Affymetrix (Santa Clara, CA, USA) to simulate the concentration of AA

in the pompano tissue. To attain the initial quantities of AAs from the practical ingredients, they were sent for composition analyses to Eurofins Scientific Inc., Nutrition Analysis Center (Des Moines, IA, USA). Each ingredient underwent proximate composition analysis for dry matter, crude protein, crude lipid, crude fiber, ash, energy, and AA profiles.

All diets were manufactured at the Fish Technology Center (Bozeman, MT, USA) by cooking extrusion. All ingredients were ground to a particle size of < 200 µm using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN, USA). The diets were processed using a twin-screw cooking extruder (DN DL-44, Buhler AG, Uzwil, Switzerland) with an 8 – 14 second exposure to 125 – 145 °C in the extruder barrel using a 5-mm die opening. Pressure at the die head varied from 32 – 47 bar, depending on test diet. Pellets were flash dried with a 4 M conveyor and cooling fan then collected and batch dried with a pulse bed drier (Buhler AG, Uzwil, Switzerland) for approximately 20 minutes at 98 °C. This resulted in final moisture levels less than 10 %, followed by cooling with forced air at ambient temperature for approximately 15 minutes. After drying and cooling periods, all added fish oil was top-coated on the feed using a vacuum coater (A.J. Mixing, Ontario, Canada). Diets were stored in plastic lined paper bags at room temperature until the beginning of the trial. Subsequently, diet samples were sent to Cumberland Valley Analytical Services, Inc. (Hagerstown, MD, USA) for AA composition analysis, total protein, lipid, ash and moisture content analysis, and gross energy content analysis (Table 3).

Table 3. Analyzed amino acid composition of experimental diets and whole-body protein content set to 43 g · 100 g diet⁻¹.

Amino acids (g · 100 g diet ⁻¹)	Diets						Whole-body protein (43 g · 100 g diet ⁻¹)
	D1	D2	D3	D4	D5	D6	
EAA							
Arginine	3.09	3.06	3.05	3.04	3.00	3.02	2.82
Histidine	1.04	1.03	1.04	1.03	1.00	1.03	1.03
Isoleucine	2.15	2.13	2.18	2.14	2.11	2.14	2.02
Leucine	3.62	3.59	3.61	3.59	3.55	3.57	3.42
Lysine	3.55	3.49	3.48	3.51	3.44	3.51	3.58
Methionine	0.52	0.71	0.92	1.11	1.29	1.56	1.28
Phenylalanine	2.03	2.03	2.03	2.01	1.99	2.00	1.94
Threonine	1.93	1.90	1.90	1.88	1.87	1.90	0.97
Valine	2.61	2.54	2.48	2.48	2.44	2.46	2.33
Tryptophan	0.71	0.68	0.69	0.73	0.71	0.71	0.72
NEAA							
Alanine	1.16	1.13	1.16	1.14	1.12	1.14	3.33
Aspartic Acid	2.77	2.62	2.55	2.46	2.33	2.28	3.60
Cystine	0.27	0.27	0.27	0.27	0.27	0.28	0.10
Glutamic Acid	4.47	4.27	4.19	4.03	3.85	3.79	5.18
Glycine	4.54	4.45	4.45	4.47	4.39	4.49	4.11
Proline	2.47	2.41	2.41	2.40	2.40	2.38	2.36
Serine	1.53	1.50	1.50	1.46	1.45	1.49	1.80
Taurine	0.71	0.70	0.71	0.71	0.71	0.72	0.48
Tyrosine	4.28	4.26	4.26	4.26	4.19	4.27	0.93

2.2 Fish and Experimental Design

The pompano used in the feeding trial were acquired from Trout Lodge (Vero Beach, FL) and shipped to the University of Miami Experimental Hatchery (UMEH) as 5 g fingerlings. They continued to be reared in a 15-m³, flow-through tank at UMEH and fed a high quality, high protein diet (Otohime, Reed Mariculture Inc., Campbell, CA, USA) until the onset of the experiment. A flow-through system consisting of 18, 1-m³ tanks was utilized for the feeding trial. Three tanks were randomly assigned per diet and stocked with 11 fish per tank averaging a total of 2290.3 ± 13.86 g initial biomass on April 12, 2015. Each tank was equipped with an oxygen stone, center standpipe for

drainage, and secured shade-cloth cover to keep fish from jumping out. Fish were acclimated to the system for a week prior to the initiation of the experiment. Before experimental diets were fed for the first time, fish were fasted for 24-hours, anesthetized with 15 ppm of 100% clove bud oil (Spice USA, Inc., Hialeah, FL, USA), weighed and measured. The initial average weight per fish was 208.2 ± 1.88 g. The trial was conducted for 60 days with 58 total feeding days.

Everyday maintenance of tanks included measuring water parameters in the morning, siphoning waste from the bottom of the tanks, and adjusting flow rates. A YSI Professional Plus Meter (YSI, Inc., Yellow Springs, Ohio, USA) was used to measure dissolved oxygen, pH, salinity and temperature. The average water temperature throughout the trial system was 27.70 ± 0.04 °C, dissolved oxygen was maintained above saturation at 8.79 ± 0.04 mg L⁻¹, salinity was 36.41 ± 0.04 ppt, pH was 8.02 ± 0.01 , and water exchange was maintained at 1500 %. Feeding occurred twice a day, once in the morning (0900 h) and once in the afternoon (1500 h) to satiation. Prior to feeding, the incoming water was shut off to prevent the loss of feed through the drainage. Any leftover feed pellets were removed and discounted from the total weight fed. On day 30 the fish were anesthetized with 100 % clove oil, weighed (g), measured (cm), prophylactically treated with a fresh water bath (0 ppt) for 2 – 5 minutes, and transferred back to their designated experimental tanks. Before the fish were transferred back, the tanks were cleaned and disinfected with Virkon S (Farnam Companies, Inc., Phoenix, AZ, USA). This procedure was done to monitor growth progress and note any physical consequences due to the experimental diets.

The experiment proceeded until day 60 and was terminated on June 11, 2015. Fish were harvested by tank and euthanized using a solution of 40 ppm of tricaine methanesulfonate (MS-222; Western Chemical, Inc., Ferndale, WA, USA) before recording weight (g). Three fish from each tank were randomly selected for hepatosomatic (HSI) and visceral-somatic (VSI) indices. These fish were weighed and dissected to attain the respective weights of the viscera and liver.

Feed Intake and Growth Performance Parameters

Several parameters were calculated to determine feed intake and growth performance for each diet. These include: Mean Daily Intake (MDI), Average Weight Gain (AWG), Weight Gain Ratio (WGR), Specific Growth Rate (SGR), Average Daily Gain (ADG), Energy Intake (EI), Protein Intake (PI), Protein Efficiency Ratio (PER), Feed Efficiency (FE), Feed Conversion Ratio (FCR), HSI, and VSI. The following are the equations for each parameter:

1. $MDI = (\text{feed intake}/\text{fish}/\text{number of days})$
2. $AWG = (\text{average final weight} - \text{average initial weight})$
3. $WGR = (\text{average final weight} - \text{average initial weight})/\text{average initial weight}$
4. $SGR = 100 (\ln \text{average final weight} - \ln \text{average initial weight})/\text{numbers of days}$
5. $ADG = (\text{average weight gain}/\text{number of days})$
6. $EI = \text{feed intake (g)} * \text{gross energy (Kcal/g dry feed)}$
7. $PI = \text{feed intake (protein \% in feed}/100)$
8. $PER = (\text{total weight gain}/\text{protein intake})$
9. $FE = (\text{total weight gain}/\text{total feed intake})$
10. $FCR = (\text{total feed intake}/\text{total weight gain})$

11. VSI= viscera weight * 100/body weight

12. HSI= liver weight * 100/body weight

2.3 Statistical Methods

To determine any significant differences among the experimental diets, all parameters were subjected to One-way Analysis of Variance (ANOVA). Prior to ANOVA, normality was verified through Shapiro-Wilk test ($P > 0.05$) and homogeneity of variance through Levene test ($P > 0.05$). Data that failed normality had a Box-Cox transformation applied. If ANOVA assumptions were not met after transformation, the Kruskal-Wallis H test was employed. If a significant difference was determined by ANOVA, a Duncan's multiple range posthoc test was used to determine differences among treatments ($P < 0.05$). All results are reported as means \pm SE. If a Kruskal-Wallis H test was used, a Dunn's posthoc test with Bonferroni adjustment was applied to determine significant differences among treatments ($P < 0.05$). The broken-line method, $Y = aX + b$ (Robbins et al., 1979), was employed to estimate the TSAA requirement from weight gain data. The program utilized for statistical analyses was IBM SPSS (22.0).

Chapter 3: Results

At the end of the 60-day trial survival for all but one tank was 100 %. One fish disappeared from tank 16 most likely due to external predation and not experimental conditions. There were no discernable trends (neither increasing nor decreasing) among growth parameters across all Met inclusion levels (Table 4). There was a significant difference in AWG ($F_{(5,12)} = 3.184, P < 0.05$) with no differences between D1, D2, D3, D5, and D6 (293.4 – 311.7); however, D4 (1.10 % Met) was significantly different with a lower AWG of 258.0 g. Similarly, ADG was also significantly different ($F_{(5,12)} = 3.187, P < 0.05$) resulting in D4 with the lowest value at 4.45 g compared to 5.06 – 5.37 g for the remaining diets. WGR (1.30 – 1.50 % initial weight) and SGR (1.43 – 1.58 % · day⁻¹) were not significantly different among the treatments ($F_{(5,12)} = 1.640, P = 0.223$ and $F_{(5,12)} = 1.749, P = 0.198$). Although performance parameters for D4 (1.10 % Met) were generally lower than the other treatments (Figure 1), there was no significant differences among all diets in MDI ($F_{(5,12)} = 1.177, P = 0.376$), PI ($F_{(5,12)} = 2.424, P = 0.097$), or EI ($F_{(5,12)} = 0.897, P = 0.513$). Additionally, there was no significant differences between treatments for FE ($\chi^2_{(5)} = 9.378, P = 0.095$), although the lowest FE value was again, D4. Significant differences were found in PER ($F_{(5,12)} = 5.785, P < 0.01$) with the highest in D6 (1.11), followed by D1 – D3, D5 (1.01 – 1.02) and D4 with the lowest mean of 0.93. There were significant differences in FCR between diets ($F_{(5,12)} = 3.626, P < 0.05$) with D1 – D4, D5, D6 having a lower FCR (2.38 – 2.50) than D4 (2.70).

Table 4. Growth performance parameters for Florida pompano (*Trachinotus carolinus*) fed six experimental diets with graded levels of methionine for 8 weeks.

Diets (Met g · 100 g diet ⁻¹)	Diet 1 0.5	Diet 2 0.7	Diet 3 0.9	Diet 4 1.1	Diet 5 1.3	Diet 6 1.5	(P > F)
AWG ¹ , (g)	311.5 ^a	298.1 ^a	311.7 ^a	258.1 ^b	293.4 ^a	302.0 ^a	0.046*
ADG ² , (g · day ⁻¹ · fish ⁻¹)	5.37 ^a	5.14 ^a	5.37 ^a	4.45 ^b	5.06 ^a	5.21 ^a	0.046*
WGR ³ , (% of initial weight)	150	146	149	130	143	143	0.223
SGR ⁴ , (% · day ⁻¹)	1.58	1.55	1.57	1.43	1.53	1.53	0.198
MDI ⁵ , (g · fish ⁻¹ · day ⁻¹)	13.24	12.67	12.94	12.01	12.64	12.36	0.376
EI ⁶ , (kcal · fish ⁻¹ · day ⁻¹)	67.02	65.00	65.96	61.73	64.08	63.23	0.513
PI ⁷ , (g protein fed · fish ⁻¹ · day ⁻¹)	5.26	5.09	5.30	4.77	4.95	4.72	0.097
FE ^{8†} , (g gain · g dry feed ⁻¹)	0.41	0.40	0.41	0.38	0.40	0.42	0.095
PER ⁹ , (g gain · g protein fed ⁻¹)	1.02 ^b	1.01 ^b	1.01 ^b	0.93 ^c	1.02 ^b	1.10 ^a	0.006*
FCR ¹⁰ , (g fed · g gain ⁻¹)	2.47 ^b	2.47 ^b	2.41 ^b	2.70 ^a	2.50 ^b	2.38 ^b	0.031*

Values are means of 3 replicate groups; different superscripts are significantly different determined by Duncan's multiple range test ($P < 0.05$).

* Denotes significance in global statistical analysis ($P < 0.05$)

† Values are medians of 3 replicate groups tested globally with Kruskal-Wallis H procedure

¹ AWG, average weight gain = (average final weight – average initial weight)

² ADG, average daily gain = (average weight gain / number of days)

³ WGR, weight gain rate = (average final weight – average initial weight) / average initial weight

⁴ SGR, specific growth rate = $100 \times (\ln \text{ average final weight} - \ln \text{ average initial weight}) / \text{numbers of days}$

⁵ MDI, mean daily intake = feed intake / fish / number of days

⁶ EI, energy intake = feed intake (g) × gross energy (kcal · g dry feed⁻¹)

⁷ PI, protein intake = feed intake (g) × (protein % in feed / 100)

⁸ FE, feed efficiency = total weight gain / total feed intake

⁹ PER, protein efficiency rate = total weight gain / protein intake

¹⁰ FCR, feed conversion ratio = total feed intake / total weight gain

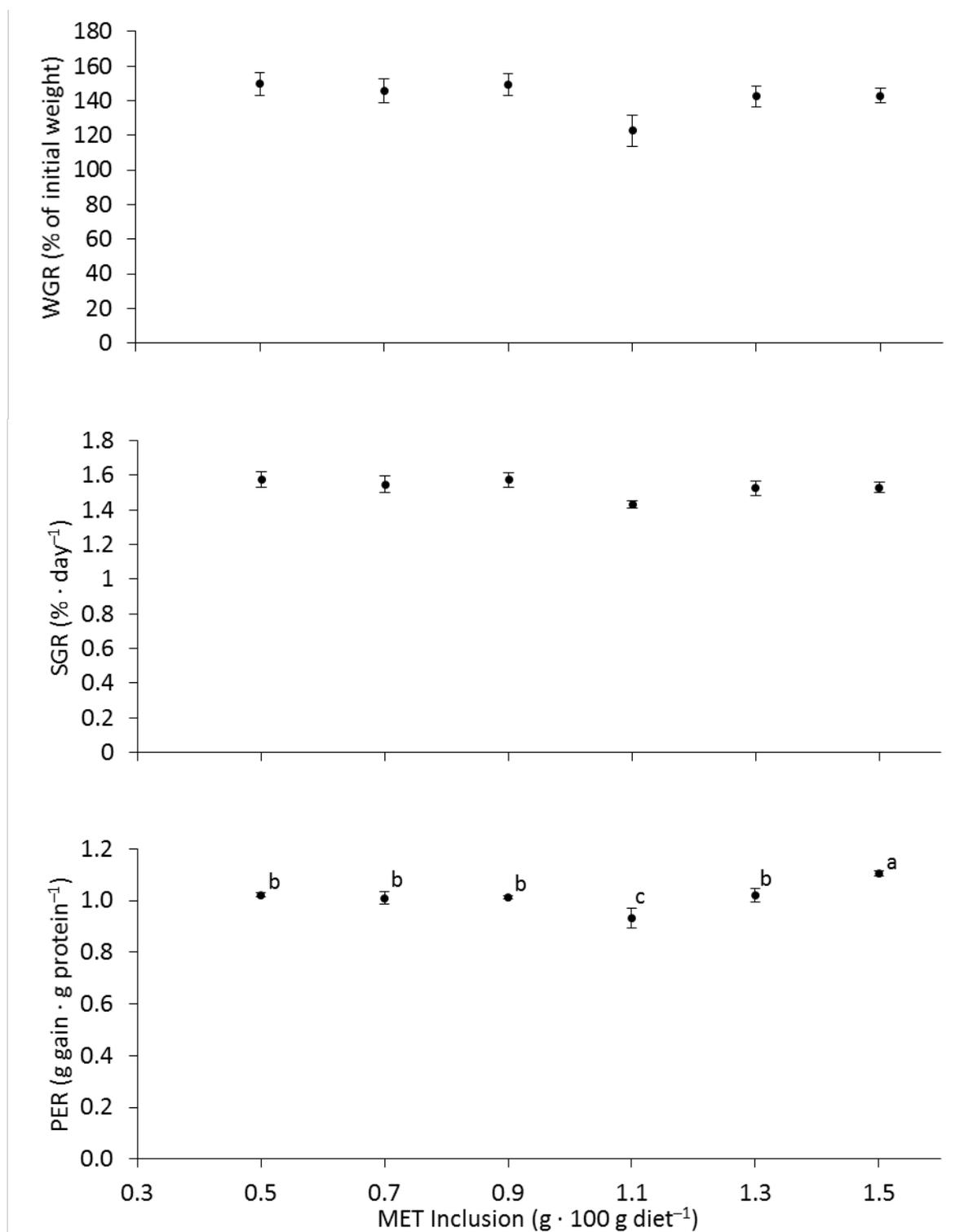


Figure 1. Means \pm SE plots of WGR (top), SGR (middle), and PER (bottom). Values are means of 3 replicate groups; different superscripts are significantly different determined by Duncan's multiple range test ($P < 0.05$).

HSI mean values were comparable for all diets with no significant differences ($F_{(5,12)} = 2.813$, $P = 0.066$) among treatments (Table 5). However, VSI was significantly different between diets ($\chi^2_{(5)} = 12.373$, $P < 0.05$) with the highest in D3 (5.30), followed by D1, D2, D4, D6 (4.29 – 4.58) and ending with D5 at the lowest (4.25).

Table 5. Mean values of HSI and VSI.

Diets (Met g · 100 g diet ⁻¹)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	(P > F)
HSI	1.59	1.57	1.74	1.45	1.41	1.61	0.066
VSI [†]	4.58 ^{ab}	4.40 ^{ab}	5.30 ^a	4.29 ^{ab}	4.25 ^b	4.88 ^{ab}	0.030*

HSI, Hepatosomatic index; VSI, Vicerasomatic index

Values are means of 3 replicate groups; different superscripts are significantly different determined by Duncan's multiple range test ($P < 0.05$).

[†] Values are medians of 3 replicate groups tested globally with Kruskal-Wallis H procedure; different superscripts are significantly different determined by Dunn's procedure with a Bonferroni correction for multiple comparisons

* Denotes significance in global statistical analysis ($P < 0.05$)

With no trend observed in growth performance among increasing Met inclusion levels, a methionine requirement could not be determined using linear regression analysis. A break point indicating the level of Met required for optimal growth was not evident (Figure 1).

Chapter 4: Discussion

Results indicated that after 60 days of culture, fish fed all diets exhibited excellent growth, comparable to commercial conditions. These results suggest that near-harvestable size Florida pompano perform well with respect to growth at the lowest methionine inclusion of $0.5 \text{ g} \cdot 100 \text{ g diet}^{-1}$. This value of methionine is below that published for *Trachinotus ovatus* juveniles at an inclusion between 1.06 and $1.27 \text{ g} \cdot 100 \text{ g diet}^{-1}$ corresponding to $2.46 - 2.95 \text{ g} \cdot 100 \text{ g dietary protein}^{-1}$ (Niu et al., 2013). The fish in the current feeding trial did not exhibit any negative effects of methionine deficiency such as bilateral cataracts or impeded growth. Signs of excess dietary methionine such as reduced growth as exhibited by rainbow trout (Poppi et al., 2011) also were not apparent in this study. Pompano from all treatments, including the lowest and highest inclusions of Met (D1, D6), exhibited normal behavior, appearance, and weight gain through the duration of the experiment. This suggests that Florida pompano can utilize practical-type diets with nearly half of dietary protein originating from crystalline AAs.

Growth performance (Table 4) of fish in the present trial was comparable to other pompano nutrition feeding trials. An earlier study conducted at UMEH with adult-sized Florida pompano (initial mean weight $247 - 288 \text{ g}$) fed practical diets of varying DP/DE ratios for 88 days resulted in a final mean weight ranging from 519 to 634 g with WGR of $110 - 120\%$, SGR of $0.84 - 0.93$, FE of $0.32 - 0.38$, PER of $0.68 - 0.76$, and FCR of $2.6 - 3.14$ (Taynor, 2013). These values are similar to those of the present study, but are generally lower for WGR, SGR, FE, and PER and higher for FCR. The difference in performance indicators could be due to the difference in initial mean weight, $247 - 288 \text{ g}$

per fish compared to 208.2 ± 1.88 g per fish for the Met trial. This suggests that a reduction in growth rate and feed efficiency could be due to increased size.

In accordance, a thesis study on the effects of feeding frequency on large Florida pompano (initial mean weight 215 g) by Groat (2002) resulted in fish weighing an average of 527 g after 63 days of feeding a commercial diet (53 % protein, 13 % lipid) twice or 4 times a day to satiation. The trial resulted in AWG between 290 – 326 g, WGR range of 135 – 151%, SGR measured at 1.3, and FE range of 0.39 – 0.41. These values are similar to those presented in Table 4. That study continued for a total of 133 days and resulted in a final mean weight of 688 – 750 g per fish. Groat (2002) noted that as the pompano increased in size, especially past 200 g, the SGR and FE tended to decline. This trend toward reduced growth from 200 g to harvest size (~ 500 g) has been the main impediment for attempts at culturing Florida pompano (Hicks, 1998; McMaster, 1988; Smith, 1973). The onset of sexual maturity could be a possible explanation due to a decrease in somatic growth and increase in gonadal growth. This highlights the importance of quantifying nutritional requirements of sub-adult and adult pompano.

Growth performance (Table 4) was also comparable or surpassed results from a 110-day growth trial on the effects of stocking density in low salinity (5 ppt) recirculating aquaculture systems on Florida pompano fed a commercial pelleted diet (50 % protein, 14 % lipid) (Weirich et al., 2009). Pompano were stocked at an initial mean weight of 259 g per fish and gained an average of 312.7 g, with an ADG of 2.8 g, FE of 0.26, and PER of 0.54. These values in addition to those from the aforementioned studies indicate that the experimental diets used in the present research were adequate for normal growth

performance and health of the pompano. This indication suggests that the optimal Met inclusion is likely lower than the lowest inclusion tested in this study (0.50 % of diet).

Past research on various organisms from humans to shrimp, corroborate the current findings. Met requirements are substantially reduced from early life stages to adult life stages in humans (Pillai and Kurpad, 2012), chickens (NRC, 1994), rats (Hartsook and Mitchell, 1956), and white shrimp (Lin et al., 2015). It is reasonable to suggest that the same trend occurs for fish, which is supported by the present data reported for pompano. This evidence is partly validated by the fact that the protein requirement of fish decreases with increasing size and age (Taynor, 2013; Wilson, 2003).

Although a specific Met requirement was not determined for adult pompano at the end of the present feeding trial, the growth performance achieved across all experimental diets was comparable to growth data from other studies on the same size fish.

Additionally, there was no evidence that any of the experimental diets compromised fish health throughout the trial period. There were no differences among treatments regarding WGR, SGR, FE, and HSI; however, D4 (1.10 % Met) values were significantly different and substandard for AWG, ADG, PER, and FCR. A logical explanation for this anomaly in growth performance would likely be differences in MDI, EI, and PI yet there were no statistical differences among the diets for those indicators. Furthermore, no major numerical differences between proximate analyses and AA composition analyses for the experimental diets were apparent. D5 and D6, which had higher inclusions of Met at 1.30 and 1.50 % respectively, performed equal to D1 – 3 and better than D4, suggesting that there were no negative effects of higher Met inclusion up to 1.5 % in the diet. Although

D4 growth performance was different, its values remain comparable to other studies on Florida pompano larger than 200 g.

Additionally, the content of the organic acid, taurine, was 0.71 % by weight across all the experimental diets. This value is higher than the amount analyzed in the whole-body AA content of the pompano ($43 \text{ g} \cdot 100 \text{ g diet}^{-1}$), which was 0.48 % by weight. Because taurine can be synthesized from Cys through the enzyme, cysteinesulphinatase decarboxylase (CSD) in fish (Yokoyama et al., 2001), the possibility of excess taurine sparing Cys and therefore, Met, exists. This would provide an explanation for the comparable growth performance among diets, especially that of Diet 1 having the lowest inclusion of Met. However, the growth performance exhibited across the experimental treatments is unlikely due to taurine inclusion in the diet considering that several carnivorous marine fish require supplementation of this nutrient in plant-based diets due to nonexistent or very low levels of CSD activity in these species (Salze and Davis, 2015; Yokoyama et al., 2001). In accordance Rossi (2011) suggested that taurine is likely indispensable for Florida pompano and Salze et al. (2014) determined a taurine requirement of 0.54 –0.65 % for the species.

The preliminary results from this investigation show that adequate weight gain can be achieved for near-harvestable-size Florida pompano using dietary Met inclusion levels as low as 0.50 % at a constant cystine value of 0.27 % of diet. Further research is needed to confirm these findings. A wider range of dietary Met inclusions, less than 0.50 % and greater than 1.50%, is suggested for experimental diet formulations in future feeding trials. It is important to quantify the optimal dietary Met inclusion for adult pompano to achieve balanced amino acid profiles that can be applied to commercial feed

formulations. Quantifying the nutritional requirements for this species would make the development of cost-effective and sustainable diets possible and thus promote commercial production.

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