UTILIZATION OF DIFFERENT DIET FORMULATIONS IN MARINE SPECIES, EFFECT OF SALINITY ON GROWTH AND SERUM OSMOLALITY OF YELLOWTAIL SNAPPER, *Ocyurus chrysurus*, AND DETERMINATION OF METHIONINE REQUIREMENT IN PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*

by

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Abstract

Marine fish and invertebrate aquaculture is a substantial source of animal protein and one of the fastest growing food sectors in the world. The current need in developing high quality and nutritionally-complete feed in promoting optimal animal growth has been the constant challenge in the aquaculture industry. Three main diets were made to determine the methionine requirement in practical diets for *Litopenaeus vannamei* – a deficient basal diet, a replete diet with DL-Met and Met-Met-supplemented diet, and a replete diet with corn protein concentrate. Ten experimental diets were produced by blending the main diets, with graded methionine levels. Significant differences were observed in weight gain and shrimp whole body amino acids. Results confirmed that in the presence of replete cystine, a conservative methionine requirement was estimated by a one-slope broken-line regression analysis model at 0.61% diet (1.68% protein) is recommended.

The present work evaluated protein sources fishmeal, poultry meal, and solvent-extracted soybean meal and determine SBM-induced enteritis in yellowtail snapper, *Ocyurus chrysurus*. Growth performance, histological measurements, and scoring of the distal intestine showed no significant differences in yellowtail snapper when fed diets containing reduced levels of fishmeal and 40% SBM. Dietary protein and lipid levels were evaluated by conducting a 14-week trial using diets with varying protein (36%, 40%, and 44%) and lipid levels (6%, 10%, and 14%), and a 10-week trial with diets having 36% protein and incremental lipid levels (7%, 10%, 13%, and 16%). We recommend 36% protein and dietary lipid levels of 7%-13%, which are lower than currently used commercial diets for marine finfish. Knowledge from the current study is helpful in formulating cost-effective feed and promote sustainable yellowtail snapper aquaculture. Salinity

tolerance in salinity conditions of 3 to 32 g/L was evaluated, followed by a six-week growth trial with salinities of 6 to 32 g/L. Significant differences were observed for survival in fish reared in 6 g/L, and for biomass, with highest weights in 12 to 16 g/L. Such findings serve as initial data in the possible potential of yellowtail snapper culture in lower salinity conditions to promote its cultivation, and open sustainable and economic mariculture approaches.

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CHAPTER 1

GENERAL INTRODUCTION

The increasing seafood demands and consequent decline in capture wild fisheries have led to the immense growth of the aquaculture industry worldwide (FAO, 2018). Marine aquatic fish and invertebrate aquaculture is now a substantial source of animal protein and has been one of the fastest growing food sectors in the world over the past 20 years (Naylor and Burke, 2005; Edwards, 2015; FAO, 2018), with an estimated first-sale value of US$106 billion (FAO, 2020). For some time, culture fish has dominated the seafood market with mariculture contributing 36% total value and 34% of total aquaculture production. Mariculture dominates in providing sustainable and nutritious food sources to help meet growing protein demand. Additionally, it also holds significant promise due to the emerging opportunities to increase production (e.g., offshore aquaculture, (Costello et al., 2020) and increasing recognition of the potentially lower environmental impacts of mariculture compared to land-based animal products (Hall et al., 2014; Hilborn et al., 2018).

Accounting for 40-60% of variable costs, feed has been and will always be considered one of the major factors affecting production costs. The current need to develop high quality feed having complete nutritional composition to promote acceptable animal growth has been a constant challenge in the aquaculture industry. However, feed development requires information on nutritional requirements at the general level to specific nutrient requirements. For example, with regards to protein one needs to understand the acceptability of various protein sources, the gross protein requirement as well as the specific amino acid (AA) requirements. Studies focusing on protein requirements often rely on determining systematic replacement of high-cost protein and the balancing of essential amino acids (EAAs) through supplementation, which provides a path for consistent reduction in high-cost protein sources such as fishmeal, while maintaining feed quality,

and meeting the nutritional requirement of the animal. Given that the protein component of the feed is quite costly, there is considerable interest in the optics of protein utilization and optimization. Hence, there is interest in identifying the efficacy of various protein sources, the response of animals to dietary protein as well as optimizing specific AA requirements. There is a current demand to develop feed formulations having complete AA information to determine specific limiting or excessive AA in the diet to establish the species AA requirement. By establishing the AA profile for each organism, diets can be formulated to be reasonably affordable and in accordance to the species' nutritional requirement without compromising nutritional value and growth. It is essential to understand basic EAA requirements to formulate nutritionally-complete and economically-feasible diets. Dietary EAA imbalance may lead to a wide variety of adverse physiological responses and, to a certain extent, may explain reduced growth seen with single-source alternate protein feeds. Complete quantitative EAA requirements are only established for a handful of the over 230 species of fish and crustaceans presently cultivated (Metian et al., 2019) making this research endeavor important in animal nutrition research. Different ways of determining EAA requirements include dose-response feeding trials which are time-consuming and expensive (Akiyama et al., 1997; Wilson, 2002). Having knowledge of the EAA requirements of a species enables formulation of feeds that reduce over-supplementation of dietary protein. By preparing feeds with the exact balance of EAA needed to promote optimum performance, low protein diets allow similar and identical performance when compared to unbalanced higher protein feeds.

The most important shrimp species in global aquaculture is the Pacific white shrimp *Litopenaeus vannamei* Boone, 1931. This species was the top species produced in 2020 that comprised 51.7% of world shrimp production (FAO, 2022b). Also known as the white-legged

shrimp, it is the most extensively farmed crustacean species in the world. The success in farming this shrimp results from its tolerance to high densities, high growth rates, and its ability to tolerate a range of water quality conditions and salinities (Briggs et al., 2004). *L. vannamei* is a Penaeid shrimp native of Pacific coast of Mexico and Central and South America as far south as Peru, where water temperatures are normally higher than 20°C throughout the year, and is mainly found on mud bottoms, down to a depth of 75 m (Rosenberry, 1998; FAO/WHO, 2002). The development and validation of fishmeal free shrimp feed formulations has been a constant challenge in the aquaculture industry. EAA requirement determination is considered to be the highest priority area in shrimp nutrition research (Akiyama, 1986). Methionine (Met) is generally the first limiting amino acid in diets formulated with high levels of plant and rendered animal by-products in shrimp feed (Qiu et al., 2017), making it necessary to supply it back through the diets in adequate amounts to meet their requirement. Various sources may be utilized to incorporate Met in shrimp diets including intact protein or chemical additives. Albeit there have been several publications looking at methionine requirement in shrimp (Millamena et al., 1996; Forster and Dominy, 2006; Nunes et al., 2014), the data is less than compelling. In several of these papers, cystine levels were not accounted for in relation to methionine levels, replication is often questionable and even within the same publication results are not always validated. When cysteine levels are not considered, the research encompasses the total sulfuric amino acid levels, and not specifically methionine alone. Furthermore, Swanepoel et al. (2022) reported on a number of studies indicating that purified sources of amino acids may not be available to shrimp. Because methionine is often a limiting amino acid in practical feed formulations having a reasonable estimate of the requirements is critical to the development of precise feed formulations. Due to the

disparity in reported values, it is necessary to establish the dietary methionine requirement of *L. vannamei.*

Marine finfish culture is of interest to aquaculturists worldwide, not only as a source of food fish but also due to stock enhancement potential for recreational and commercial fisheries. More specifically, snapper are a popular species among commercial and recreational fisheries due to their diversity and abundance along the Gulf of Mexico and the Atlantic Ocean. This fish species is known to be long-lived, highly fecund, and are typically attain sexual mature after 2 years. However, juveniles suffer from variable survival rates in year 1 (~86% in first year) and year 2 (~70%) (Gallaway et al., 2009; Phelps et al., 2009; Gallaway et al., 2020). These fish feed on small fish and crabs (Usman et al., 2014). The diet of *Lutjanus guttatus* and *L. peru* in different areas of the Pacific coast of Mexico has revealed consumption of crustaceans, mollusks, and fish (Tripp-Valdez and Arreguín-Sánchez, 2009; Torres-Rojo et al., 2016). These marine fish command a high price in international and local seafood markets because of their taste, quality, and consumer preference (Sivaraman et al., 2019). Moreover, snapper species are considered important for artisanal tropical water fisheries worldwide, due to the quality of their meat and commercial value (Arreguín-Sánchez and Manickchand-Heileman, 1998). These fish are captured in the Mexican Pacific along with several target species, such as the spotted rose snapper, Pacific red snapper, and yellow snapper (*L. argentiventris*), among others (Ramos-Cruz, 2001). The snapper fishery industry occurs year-round with some variations, as this resource is in high demand for food and attains a good price in the market (Ramos-Cruz, 2001). In addition to their commercial importance, snapper plays an important ecological role, participating actively in the energy flow of trophic webs, and are considered linking species (Arreguín-Sánchez and Manickchand-Heileman, 1998; Del Mar Quiroga-Samaniego et al., 2022). However, with the recent developments in hatchery

techniques to produce sufficient fingerlings and juveniles, snapper culture has sparked interest in commercial production (Ibarra-Castro and Alvarez-Lajonchère, 2011). In fact, De la Guardia et al. (2018) reported that snapper populations in several regions have declined due to overfishing. It is often caught by anglers at a smaller size or as a mature broodfish without spawning, hence wild populations are threatened. The decline in fish populations is also attributed to climate change (Glaser et al., 2019; Parsons et al., 2020)

Current studies evaluating the potential of the yellowtail snapper *Ocyurus chrysurus* (Bloch, 1791) and its culture poses a promising reception and positive potential for development in the aquaculture industry. This species is known to have a high market value and supports an economically-important commercial and recreational fishing industry (Ibarra-Castro et al., 2013). Yellowtail snapper is widely distributed in the tropical and subtropical western Atlantic Ocean from the United States to Brazil (De La Morinière et al., 2003) and are considered a desirable species in the Caribbean Islands (Soletchnik et al., 1989). This marine fish is an important fisheries resource and is considered a good saltwater species candidate for aquaculture due to advantages in non-hormonal spawning, handling tolerance, artificial feed acceptance, and good consumer reception (Watanabe et al., 1998; Turano et al., 2000; Gutiérrez-Sigeros et al., 2018). Moreover, it also serves as a conservation species to help rebuild wild populations through stock enhancement programs and potentially as a high value food species to supplement commercial fishery harvests (Saillant et al., 2013). Grow-out trials with juveniles from the wild, have indicated good adaptation to breeding conditions, rapid weaning and high survival rates (Thouard et al., 1990).

Information on nutrition and culture studies on the yellowtail snapper are limited. Although there is considerable interest in developing culture techniques for snapper since it is considered an easy species to culture, attempts to determine essential fatty acids (EFA) requirements have been

met with limited success (Davis et al., 1998). One key to successfully commercial aquaculture adoption of a species is being able to provide a suitable diet. However, this is dependent on information on the species preference of ingredients as well as nutritional requirements. In the absence of specific requirement data, diets for other species are often utilized. In the case of marine finfish, these diets are commonly based on information available in the salmonid industry. Typical marine fish feeds contain from 45 to 50% protein and from 10 to 20% lipid, making such feeds expensive for culture production and potentially inappropriate for slower growing species and those that are not as tolerant of higher dietary lipid levels. It is critical to know the nutritional needs of cultured species, which often means starting with basic nutritional needs. This way, producers have better guidance and knowledge on proper feed selection, promoting best growth performance, and production costs. Therefore, determination of dietary protein and lipid tolerance as well as acceptance of major ingredients should be a priority. The possible use of lower protein and lipid levels for feeds may promote cheaper feed formulation alternatives and help reduce feed costs. In addition, the possible use of different protein sources aside from fishmeal may also promote affordable options in making feeds for high-value marine fish at a reasonable price.

Aside from feeds, another major cost for aquaculture production, specifically for mariculture, is the cost of artificial sea salt. The need for full strength seawater limits the number of viable farm sites and/or increases costs when running inland RAS systems. Coastal and inland aquaculture sites can experience rapid increase and reduction in salinity due to evaporation or inundation with rain, respectively. Therefore, alternative coastal and inland sites including the use of saline groundwater needs to be evaluated for their potential for snapper farming (Fielder et al., 2002). The ability of a fish species to tolerate a wide salinity range offers more opportunity for farmers to utilize sites and saline water from a number of sources, compared with a species that is

relatively stenohaline (Fielder et al., 2007). Salinity can be a major source of stress in fish, causing osmotic imbalances which may ultimately lead to death upon the inability of the fish to maintain proper osmotic and ionic levels. At sub-lethal levels, osmotic stress resulting from salinity can cause chronic low-level stress and inhibit growth (Cotton et al., 2003; Rahmah et al., 2020), increase disease susceptibility (Cuesta et al., 2005; Resley et al., 2006; Imsland et al., 2008), and cause physiological changes in fish (Deane and Woo, 2009). Fundamental to the selection of a fish species for culture in a particular environment is an understanding of the physiology of the fish and the adaptations which may or may not occur in response to challenge with changes in the environment (Fielder et al., 2007).

To date, there is no data on the salinity tolerance of the yellowtail snapper. However, a wide variety of salinity acclimation rates per hour have been used for snapper culture from 0.08 g/L for Pacific red snapper, *Lutjanus peru* (Castillo-Vargasmachuca et al., 2013), 0.21 g/L for gray snapper, *Lutjanus griseus* (Wuenschel et al., 2004), 0.42 g/L for schoolmaster snapper, *Lutjanus apodus*, (Trehern et al., 2020) to abrupt salinity changes of 15 g/L for Australian snapper, *Pagrus auratus* (Fielder et al., 2007) to 24 g/L for mangrove red snapper, *Lutjanus argentimacultus* (Estudillo et al., 2000). The maintenance of safe rearing conditions is important for the health and well-being of cultured organisms, but rearing organisms at unnecessarily high salinities can increase the costs of production (Galkanda-Arachchige et al., 2020), and may limit production to only coastal areas or areas where a high salinity effluent could be returned to the environment without degrading local water quality. Salinity is an important environmental variable affecting the growth, survival, and feed intake of fishes (Arunachalam and Reddy, 1979; Lambert et al., 1994; Boeuf and Payan, 2001). The growth of euryhaline fish can be improved by rearing them at salinities that expend minimal energy for osmoregulation (Sampaio and Bianchini, 2002; Antony

et al., 2021). Evaluation of the viability of yellowtail snapper culture at considerably lower salinities than seawater may be a sustainable and cost-effective strategy for culture. In indoor production systems where salt mixtures may represent about 15% of total costs, a reduction in expenditures on salt may promote reasonably economical mariculture production (Fleckenstein et al., 2022). Moreover, the possible culture of the yellowtail snapper under low salinity conditions may promote inland culture, specifically in areas that have limited access to seawater. Limited studies on the yellowtail snapper constitutes the need to determine the isosmotic point for its salinity tolerance, which represents the point where the osmolality of the blood and water is equal, and osmoregulation is energetically minimal (Boeuf and Payan, 2001).

Overall objectives of these studies

Generally, the present study is aiming to establish base-line data on practical feed formulations for marine organisms including the Pacific white shrimp, *L. vannamei* and yellowtail snapper, *O. chrysurus.* Given the necessity to use alternative feed ingredients in commercially important marine species with optimal amount of amino acid composition, the present study aims to determine the dietary methionine requirement in Pacific white shrimp by using three different Met sources (Lentil meal, Met-Met, and Corn protein concentrate). Studies exploring yellowtail snapper culture are insufficient, hence knowledge related to husbandry and culture of this species is poorly understood. The objectives of this study include:

1. Determine the general specification of protein and lipid levels of practical diets (possible effects on the growth response utilizing experimental diets with varying protein and lipid concentrations, in comparison to a commercial diet)

2. Evaluate the use of lower cost alternatives to fishmeal (namely fishmeal, poultry meal, and soybean meal)
3. Determine the salinity tolerance and osmoregulation of the yellowtail snapper, as there is interest in culturing these species in RAS systems for which reduced salinities would have economic advantage.

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CHAPTER II

THE USE OF INTACT PROTEINS AND PURIFIED AMINO ACIDS IN DETERMINING THE METHIONINE REQUIREMENT IN PRACTICAL DIETS OF PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*

Abstract

As there is no consensus on the efficacy of purified amino acids in shrimp feed, two approaches were conducted to evaluate the methionine requirement in practical diets for *Litopenaeus vannamei*. The first approach used intact proteins to produce both a deficient and a replete diet. These diets were then co-mixed to create varying levels of methionine using intact protein. The second approach was to supplement the basal diet with pure methionine to create a replete diet and again co-mix the two diets to produce different levels. Hence, three main diets were made, which included a deficient basal diet (B, 0.48% methionine), a replete diet (M, 0.85% methionine) that used DL-Met for the first trial, and a dipeptide Met (Met-Met)-supplemented diet for the second trial, and a replete diet (C, 0.84% methionine), which contained corn protein concentrate to increase the level of methionine. Ten experimental diets were produced by blending the deficient diet with the replete diet which resulted in graded levels of methionine namely B100, B70:M30, M100, B90:C10, B80:C20, B70:C30, B60:C40, B40:C60. B20:C80, and C100. Test diets were then fed to shrimp (15/aquaria) in 60 randomly-assigned aquaria (55.8 L) with a mean initial weight of 0.45 ± 0.002g over a 54-day growth trial (Trial 1) and 0.23 ± 0.0001g over a 42-day growth trial (Trial 2). All diets were formulated to be isonitrogenous and isolipidic (36% protein and 8% lipid, as is), with the basal diet formulated with fishmeal and lentil meal as the

primary protein sources and whole wheat as a carbohydrate source. Significant differences were observed in weight gain as well as shrimp whole body amino acids namely alanine, arginine, glycine, histidine, phenylalanine, proline, and taurine for Trial 1 and cysteine, glycine, threonine, and taurine for Trial 2. The optimal dietary methionine requirement of *L. vannamei*, estimated by a one-slope broken-line regression analysis model based on weight gain% was 0.67% of the dry diet (equivalent to 1.85% of dietary protein on a dry-weight basis) for Trial 1 and at 0.56% of the dry diet (equivalent to 1.55% of dietary protein on a dry-weight basis) for Trial 2. For thermal growth unit coefficient the optimal dietary methionine requirement of *L. vannamei*, estimated by a one-slope broken-line regression analysis model was at 0.66% of the dry diet (equivalent to 1.82% of dietary protein on a dry-weight basis) for Trial 1 and at 0.61% of the dry diet (equivalent to 1.68% of dietary protein on a dry-weight basis) for Trial 2. Findings from these trials confirm that in the presence of replete cystine that a conservative methionine requirement of 0.61% diet (1.68% protein) is recommended.

KEY WORDS: Aquaculture, Pacific white shrimp, methionine requirement

1. Introduction

The most important shrimp species in global aquaculture is the Pacific white shrimp *Litopenaeus vannamei* Boone, 1931, which comprises over 52% of production (FAO, 2022c). Also known as the white-legged shrimp, it is the most extensively farmed crustacean species in the world. The global success of farming this species is related to its tolerance to high densities, high growth rates, and its ability to tolerate a range of water quality conditions, particularly a wide range of salinities (Roy et al., 2010; Pimentel et al., 2023). *L. vannamei* is a Penaeid shrimp native to the Pacific coast of Mexico and Central and South America as far south as Peru, where water temperatures are normally higher than 20°C throughout the year, and is mainly found on mud bottoms, down to a depth of 75 m (Rosenberry, 2002; FAO, 2003). As in previous years, marine shrimp continued to dominate crustacean aquaculture, with shrimp production in 2022 reaching 1,087,111 mt (66.0% of global crustacean aquaculture production) and valued at US$6,880,068,900 (73.4% of total value) (McIntosh, 2022). Aquaculture currently provides just over a quarter (26.1%) of total global shrimp supply. Shrimp aquaculture is a rapidly growing industry with a total production in million metric tons each year (Anderson et al., 2016). In spite of success of white leg shrimp culture, some production challenges include high susceptibility to certain pathogens including Taura Syndrome Virus (TSV), White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Infectious Hypodermal Haematopoietic Necrosis Virus (IHHNV) and Lymphoid Organ Vacuolization Virus (LOVV) among others. *L. vannamei* also has high vulnerability to hypoxia, and a high risk of environmental impacts due to its high-density culture in intensive aquaculture management.

With regards to commercial feeds, the development and validation of fishmeal free shrimp feed formulations has been a challenge in the shrimp aquaculture industry but has been

demonstrated across a range of production systems. However, to optimize feeds it is critical to determine and verify essential amino acid (EAA) requirements. While there are published estimates of the requirements, there is still a need to optimize and validate the specific level of certain EAAs, specifically methionine. Moreover, there is continued pressure to reduce feed costs which is often driven by the cost of protein sources, making the over supplementation of protein or EAA impractical. This has resulted in renewed interest in AA research as a priority for shrimp nutritionists.

Methionine (Met) is an essential sulfur-containing AA which has been widely considered the first limiting amino acid for aquatic animal feed formulations when replacing fishmeal with relatively inexpensive plant and terrestrial animal proteins (Gatlin et al., 2007; Hardy, 2010; Qiu et al., 2017). In addition to mixing intact protein (IP) sources, various purified sources could be utilized to reduce feed cost. There are various forms of Met that are widely available commercially, such as DL-Met, L-Met, Met-Met (AQUAVI®, dipeptide DL-methionyl-DL-Methionine (Guo et al., 2020), microencapsulated Met, and methionine hydroxyl analogue calcium or DL-2-hydroxy-4-methylthiobutanoic acid (Forster and Dominy, 2006; Nunes et al., 2014) among others. These different Met forms are some examples of purified crystalline amino acids (CAA) that are supplemented to diets to improve the AA balance and optimize dietary profile, and consequently improve growth and protein synthesis (Nunes et al., 2014).

There are still some concerns with using synthetic AAs in aquatic animal diets, especially for shrimp, with its slow feeding behavior and external mastication of the feed (Ji et al., 2021). There are numerous studies that have demonstrated the effective utilization of synthetic AAs by aquatic animals specifically the black tiger shrimp (*Penaeus monodon*) (Niu et al., 2013), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Li et al., 2009a), and Atlantic salmon (*Salmo*

salar) (Espe et al., 2014). However, there are still doubts related to the use of CAAs in aquaculture nutrition as it has been documented to be excreted from fish gills (Richard et al., 2010), water soluble compounds leach from the feed which is especially problematic in slow-eating species like the shrimp, as well as concerns with the faster uptake or asynchronous absorption when compared to IP sources.

DL-methionine is a commonly used Met additive and has been established to replace the natural, protein derived, L-methionine in fish diets across a range of species (Goff and Gatlin, 2004; Li et al., 2009b; Powell et al., 2017). The dipeptide DL-Methionyl-DL-Methionine (AQUAVI® Met-Met, Met-Met) produced by Evonik Operations GmbH (Hanau, Germany), is a novel Met dipeptide, consisting of four different Met stereoisomers (DL-Me-Met, LD-Met-Met, DD-Met-Met, and LL-Met-Met), and is shown to be efficiently cleaved by digestive enzymes of crustacean and fish to form free D- and L-Met. Furthermore, AQUAVI®Met-Met has characteristics such as lower water solubility than other Met sources which can help to overcome the challenges related to leaching. In addition, several preliminary studies have suggested that Met-Met has a higher bioavailability than other Met sources in fish and shrimp (Niu et al., 2018; Xie et al., 2018; Wang et al., 2019; Guo et al., 2020). Contrary to these findings, various studies reported a wide range of Met levels, which did not show positive results (Millamena et al., 1996; Lin et al., 2015; Façanha et al., 2018). There are a few studies that have proposed the Met requirement to be 0.7%-0.9% of the diet for *Penaeus monodon* (Millamena et al., 1996; NRC, 2011; Façanha et al., 2018; Nunes et al., 2019). Unfortunately, some of these studies rely on limited data with no validation of the results, some do not consider low Cys levels which would increase the methionine requirement and/or more appropriately describe a TSAA requirement. Met is required for cysteine (Cys) synthesis as its metabolic precursor, and any substitution by Cys for dietary Met requirement

can only be via inhibition of the sulfur amino acid pathway that leads to synthesis of the transsulfuration metabolites, including cysteine itself (Ball et al., 2006), therefore it is important to consider Cys levels due to its sparing effect on Met. It is clear that there is disagreement over the use of various Met supplements, which have often been inconclusive or appear to be inconsistent due to species-specific differences or lack of bioassay sensitivity (Niu et al., 2018).

Feed formulators typically rely on blending protein sources to provide a suitable AA balance to their feeds albeit pure sources are also used. Different studies conducted in fish such as rainbow trout (*Oncorhynchus mykiss*) and hybrid striped bass have demonstrated that AA in IP sources are utilized more efficiently than those provided in crystalline form (Sveier et al., 2001; El Haroun and Bureau, 2007; Dabrowski et al., 2010). Hence, another way to approach a requirement is to blend protein sources with different EAA profiles. For example, the production of highly digestible corn protein concentrate (CPC) with added benefits of EAA such as lysine within a single ingredient is now feasible. CPC is a product fortified with dried lysine (some having fermentation products), and contains high levels of arginine, tryptophan, threonine, and other AAs (Khalaji et al., 2016). Interestingly, the use of CPC in supplementing EAAs, specifically lysine in diets for white shrimp has been proven comparable with crystalline lysine supplementation (Yu et al., 2013). Given the compelling attributes of CPC as an intact protein source, there is considerable interest in its utilization for EAA supplementation, and possible comparison to other crystalline Met sources such as Met-Met and DL-Met.

It is necessary to establish the dietary methionine requirement of Pacific white shrimp, while utilizing different Met sources for optimal animal growth. Met sources can be compared to understand the relative ability of these products to meet the Met requirement (Dilger and Baker, 2007). To properly use Met sources, knowledge concerning its biological effectiveness and effects

on shrimp growth and composition, as well as simultaneous dose-response were performed. Moreover, there are insufficient data regarding the comparison of different Met sources such as DL-Met, Met-Met, and an intact protein source such as CPC supplementation on growth performance, nutrient retention, and Met requirement determination of Pacific white shrimp. Given the necessity to use alternative feed ingredients in commercially important shrimp species with adequate amounts of AA, the present study aimed to determine the dietary Met requirement of Pacific white shrimp by using three different Met sources (DL-Met, Met-Met, and CPC) through the analysis of growth performance, nutrient retention, and a dose-response trial using a broken line model approach.

2. Materials and Methods

2.1 Experimental Design and Diets

As there is no consensus on the efficacy of purified amino acids in shrimp feed, both intact protein and purified supplements were utilized. The first used intact proteins to produce a deficient and a replete diet utilizing lentil meal (0.41 % methionine and 0.53 % cysteine) and CPC (1.94% methionine and 1.41 % cysteine) as the primary protein source, respectively. The second approach used the deficient diet supplemented with a high level of pure Met, using DL-Methionine for Trial 1 and Met-Met for Trial 2. Hence, three main diets were produced, including a deficient basal diet (Basal), a Met-supplemented diet (Basal+DM for Trial 1 and Basal+MM for Trial 2), and a replete diet (Basal+IP). The basal diet was then co-mixed at different ratios with the replete diets to produce graded levels of Met. Since cystine was greater than 40% of the TSAA (Total Sulfur Amino Acids), this dose response would represent the methionine requirement with cysteine in slight excess. Ten experimental diets were produced which were then fed to shrimp in 60

randomly-assigned aquaria (55.8 L) stocked with 15 juvenile shrimp each. The experimental system was connected to a common reservoir sump (1019.4 L), bead filter, fluidized biological filter (625.9 L), and recirculation pump (66.4 L/min flow rate). The total volume of the experimental system is 4993.3 L. All diets (Table 1) were formulated to be isonitrogenous and isolipidic (36% protein and 8% lipid, as is).

All experimental diets were produced at the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA) using standard procedures for shrimp feeds. Briefly, diets were prepared by mixing the pre-ground dry ingredients in a food mixer (Hobart, Troy, OH, USA) for 10–15 minutes. Boiling water was blended into the mixture to obtain a consistency appropriate for pelleting. Diets were then pressure-pelleted using a meat grinder with a 2.5-mm die. The wet pellets were placed into a forced air oven (< 45 °C) overnight to attain a moisture content of less than 10%. A portion of each diet was ground and sieved prior to use in the earlier stages of the study, until the shrimp were large enough to consume a 2.5 mm pellet. All diets were stored in a freezer at -20°C until used in the experiment. The primary ingredients and diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate and AA composition (Table 2 and Table 3 for Trials 1 and 2, respectively).

2.2 Growth trial

The growth trial was conducted at E.W. Shell Fisheries Center at Auburn University, Auburn, Alabama in an indoor clear-water recirculation system consisting of sixty 75-L glass aquaria connected to a common reservoir tank (800-L) in a temperature-controlled environment. Pacific white shrimp were obtained from Sun Shrimp, American Mariculture, Inc., Pine Island, Florida, USA (Trial 1) and Home Grown Shrimp USA, Indiantown, Florida, USA (Trial 2).

Juvenile shrimp were reared in a nursery system and offered commercial feeds (Ziegler PL raceway Plus, 50% CP and 15% crude fat, and later shrimp starter diet 55% CP and 15% fat) until shrimp have reached an appropriate size for stocking. Dechlorinated city water was used for backwash and make-up water, and reconstituted sea salt (Crystal Sea Marinemix, Baltimore, MD, USA) dissolved with dechlorinated fresh water from Water Works Board of the City of Auburn (salinity 0.1 g/L, pH 7.3, alkalinity 29 ppm). Mechanical and biological filtration were utilized, with each tank receiving supplemental aeration via a regenerative blower and air stones supplied to individual tanks.

During the growth trial, six replicate groups per dietary treatment were randomly assigned and shrimp were fed 4 times per day. Fifteen juvenile shrimp were hand-sorted to uniform size and stocked into each aquarium tank in the experimental system. Mean initial weight for Trial 1 was 0.45 ± 0.002g and 0.23 ± 0.0001g for Trial 2. Shrimp were counted weekly to adjust daily feed allocation, and daily feed ration was calculated based on expected growth assuming a feed conversion ratio of 1.8 and doubling in size (approximately every 7 days) until the estimated shrimp weights were in excess of 1 g. Shrimp were counted once a week to assess survival and to adjust daily feed input. At the end of the growth trial, shrimp were counted and group weighed to determine mean final biomass, final weight, survival, and feed conversion ratio (FCR) according to standard calculations (NRC, 2011). Upon termination of the trial, shrimp were weighed and counted and 5 individuals per tank were randomly selected, packed in sealed bags, and stored in a freezer (-20°C) for chemical analysis. Percentage weight gain (WG%) was calculated to estimate tissue replacement, while thermal-unit growth coefficient (TGC) was calculated to estimate growth rates (Iwama and Tautz, 1981).

$$\mathrm{WG\%} = \frac{\mathrm{FBW} - \mathrm{IBW}}{\mathrm{IBW}} \times 100$$

$$\mathrm{TGC} = \frac{FBW^{1/3} - IBW^{1/3}}{\sum_d T} \times 100$$

2.3 Water Analysis

Dissolved oxygen, salinity, and water temperature were measured twice daily using a YSI-55 digital oxygen/temperature meter (YSI corporation, Yellow Springs, Ohio, USA), and total ammonia N (TAN) and nitrite-N were measured twice per week using YSI 9300 photometer (YSI, Yellow Springs, OH). The pH of the water was measured twice weekly during the experimental period using a pHTestr30 (Oakton Instrument, Vernon Hills, IL, USA). In Trial 1, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 7.12 ± 0.06 mg/L, 28.46 ± 0.05 ºC, 4.95 ± 0.10 mg/L, 7.57 ± 0.13, 0.77 ± 0.27, and 0.09 ± 0.02, respectively. While DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.86 ± 0.04 mg/L, 31.69 ± 3.18 ºC, 7.05 ± 0.09 mg/L, 7.13 ± 0.15, 0.22 ± 0.05, and 0.22 ± 0.08, respectively in Trial 2.

2.4 Statistical analysis and analytical model

All data were analyzed using SAS (V9.4, SAS Institute, Cary, NC, USA). Growth indices of shrimp were analyzed using one-way ANOVA to determine significant differences ($p < 0.05$) among treatments followed by Tukey's multiple comparison test to evaluate significant differences between treatment means. Regression analysis was used to determine the relationship between growth parameters of shrimp and dietary Met levels. Weight gain (%) and TGC were fitted against dietary Met levels using the broken-line analysis model (BLM) to estimate the quantitative Met requirement. BLM, fitted by the method of least squares, yields an objective estimate of the level of dietary nutrient that is fully adequate, also called the nutrient "requirement" (Robbins, 1986).

This model defines the requirement as the abscissa of the breaking point between a linear ascending portion of the response line and a linear portion (plateau) (Guo et al., 2020). Mathematically, the model with a one-slope broken line is described as follows:

$$y = L + U * (R - x_{LR})$$

Where y is the dependent variable, L is the ordinate and R is the dietary methionine level or the abscissa of the breakpoint in the curve, where $(R - x_{LR})$ equals 0 when $x_{LR} > R$ and $(x_{LR} - R)$ equals 0 when $x_{LR} < R$, L is an asymptote of the quadratic ascending segment of the model. U is the slope of the line for $X < R$, in which $(R < X_{LR})$ is zero when $X > R$ (Robbins, 1986). A linear model was fit to the experimental data for weight gain (%) and TGC by means of the NLIN procedure in SAS (SAS Institute, 1989) to iteratively adjust the initial parameter estimates.

3. Results

3.1 Growth performance indices

For the first trial, significant differences were observed across all treatments of shrimp fed different methionine and intact protein sources (Table 4). The highest biomass was observed in shrimp fed B40:C60 (0.63 methionine level), while the lowest biomass was observed in treatment B70:DM30 (0.51 methionine level). Weight gain (%) was highest in treatment B0:C100 (0.75 methionine level) while the lowest was B70:DM30 (0.51 methionine level). Highest survival was observed in shrimp fed B70:DM30 (0.51 methionine level), while lowest survival was observed in shrimp fed B20:C80 (0.72 methionine level). Highest values for TGC were observed in shrimp fed B0:C100 (0.75 methionine level), while the lowest was observed in shrimp fed B70:DM30 (0.51 methionine level).

In the second trial, significant differences were observed in final weight and weight gain (Table 5). The highest final weight and weight gain were observed in shrimp fed B20:C80 and B0:C100, at 0.65 and 0.72 methionine levels, respectively, which were not significantly different from each other, but were significantly different compared to all other dietary treatments. The lowest final weight and weight gain were observed in shrimp fed B70:MM30 (0.54 methionine level).

When comparing both trials, B0:C100 (0.75 methionine level) showed significantly better weight gain (%) and TGC across all dietary treatments while in the second trial, B20:C80 (0.65 methionine level) and B0:C100 (0.72 methionine level) showed significantly better final weight and weight gain. Consequently, B70:DM30 (0.51 methionine level) showed the poorest growth performance, except for survival in the first trial, while B70:MM30(0.54 methionine level) had the poorest performance in the second trial.

3.2 Shrimp proximate and amino acid composition

No significant differences were observed among all proximate composition parameters such as crude protein, moisture, crude fat, crude fiber, and ash among all dietary treatments for either growth trials (Table 6). For the first trial, significant differences were observed in whole-body shrimp AA profile for alanine, arginine, glycine, histidine, phenylalanine, proline, and taurine. It can be observed that for the AA alanine, experimental diets containing DL-Met sources did not have significant differences among each other, as well as the basal diet and IP sources when grouped together. While for arginine, all experimental diets were similar, except for B70:C30 and B0:C100, which are interestingly from low and high IP inclusion treatments respectively.

For the second trial, significant differences were observed in whole-body shrimp AA profile for cysteine, glycine, taurine, and threonine (Table 7). For cysteine, it was observed that experimental diets containing Met-Met sources did not have significant differences among each other but were significantly different when compared to all other experimental treatments. For glycine, all experimental treatments were almost similar, except when compared to B70:MM30, as well as for both B20:C80 and B0:C100 (which are both from the highest IP inclusion treatments, respectively). For taurine, similar values were observed for B20:C80 and B0:C100, which are from the highest IP inclusion treatments, while all other treatments were significantly different among each other. In contrast to threonine, all treatments showed similar values except for B80:C20 and B40:C60. Taurine showed significantly different results for both trials, suggesting that utilizing different methionine sources such as crystallized amino acids and intact proteins may alter taurine levels in shrimp.

3.3 Nutrient retention

Significant differences were observed for protein, methionine, and lysine retention values, for all dietary treatments in both trials (Table 8). For Trial 1, the highest protein retention was observed in shrimp fed B0:C100, while the lowest was with shrimp fed B70:M30. Met retention was highest with shrimp fed B90:C10, and lowest in shrimp offered M100, and the highest and lowest lysine retention were observed in shrimp fed B0:C100 and B70:M30, respectively. In contrast, in Trial 2, the highest protein retention was observed in shrimp fed B0:C100, while the lowest was observed in shrimp fed B70:M30(similar to Trial 1). Met retention was highest in shrimp fed B80:C20, and lowest in shrimp offered B70:M30. Lastly, the highest and lowest lysine retention was observed in shrimp fed B0:C100 (similar to Trial 1) and the basal diet, respectively.

4. Discussion

The blending of intact protein sources and the supplementation of CAA is a common practice to cope with the dietary imbalance in AA composition of feed for fish and shrimp (Biswas et al., 2007; Kader et al., 2012) that is primarily caused by the utilization of high levels of low cost proteins that are low in EAAs. Although dependent on the matrix of protein sources, methionine and lysine are often the first limiting AAs, resulting from lower digestibility and impaired nutrient retention efficiency (Lim and Dominy, 1990; Paripatananont et al., 2001; Biswas et al., 2007; Kader et al., 2010; Rahman et al., 2010; Bulbul et al., 2015). For this work we concentrated on looking at the methionine requirement as this often more limiting that lysine.

4.1 Growth performance

In the present study, different responses were observed in shrimp growth performance when fed with diets having CAA or IP supplementation for both trials. As compared to the response to intact protein, there was a very limited response observed when shrimp were fed with CAA sources DL-Met or Met-Met. Such insufficient CAA response was demonstrated by Deshimaru (1976) which resulted in poor growth and survival of shrimp, which agrees with the work of Swanepoel et al. (2022) that questioned whether CAA were efficiently utilized by white shrimp. Poor shrimp performance caused by high CAA supplemented diets may be attributed to leaching from the feed, rapid absorption, and uncoordinated assimilation in tissues (Deshimaru, 1976). Reduced overall fish growth performance has also been reported in fish species such as channel catfish after CAA supplementation when replacing IP from 32% to 24% (Salem et al., 2022).

There is no simple answer on the use of CAA supplements in aquatic animals as there are also numerous reports of positive results. The mentioned studies are in contrast to the results of others which may, as will all studies, have design flaws such as limited replication, limited number of treatments in the dose response or limited growth resulting in low levels of tissue replacement. Niu et al. (2018) using the same Met sources (DL-Met and Met-Met) in white shrimp reported on improved bioavailability of the Meth-Meth product for which the responses are different albeit growth or tissue replacement is relatively low. Swick et al. (1995) working with the black tiger shrimp reported improved growth, final weight, and feed conversion with high soybean, low fishmeal diets supplemented with HMTBA (2-hydroxy-4-methylthiobutanic acid) or DL-methionine supplementation for 9 weeks. Other studies confirming improved growth response of Met supplementation against Met deficiency in white shrimp have been conducted (Millamena et al., 1996; Forster and Dominy, 2006; Chi et al., 2011; Gu et al., 2013). A positive growth performance with increasing Met supplementation was also observed in fish such as the sunshine bass (*Morone chrysops* × *Morone saxatilis*) (Keembiyehetty and Gatlin III, 1995), red sea bream (Mamauag et al., 2012) and channel catfish (Robinson et al., 1978). On the contrary, a study by (Ji et al., 2021) reported that Met-Met supplementation could significantly mitigate poor growth and adverse effects caused by low fishmeal diets offered to *L. vannamei.* Albeit lower and upper responses to methionine levels in shrimp can be identified, most of these studies demonstrate efficacy, however, these do not identify a requirement using a dose response model.

In this study, IP was used to raise the methionine level, shrimp performed better as methionine from CPC increased, which is demonstrated in weight gain (%), FCR, and TGC for both Trial 1 and Trial 2. Such results with IP utilization for AA supplementation have been observed in both fish and shrimp. For instance, the use of IP is reported to be more effectively

utilized than CAA sources in channel catfish (*Ictalurus punctatus*) (Nguyen and Davis, 2016), rainbow trout (*Oncorhynchus mykiss*) (El Haroun and Bureau, 2006), and Atlantic salmon (*Salmon salar*) (Sveier et al., 2001; Hauler et al., 2007). High lysine CPC was also reported to improve growth performance to a level comparable when CAA lysine sources when supplemented in tilapia diets (Nguyen and Davis, 2016). Moreover, the use of lysine-enhanced CPC was proven to be comparable to lysine CAA for shrimp *Litopenaeus vannamei* (Yu and Zhang, 2012).

Although not observed in this work, in some cases, a decline in weight gain is reported as crystalline supplements are used at high levels. For example, (Millamena et al., 1996; Lin et al., 2015) reported a decline in weight gain and SGR with higher supplementation levels of dietary methionine in white shrimp. A significant decrease in growth performance was also reported in kuruma shrimp, *Marsupenaeus japonicus* when fed with diets containing less than 0.80 and 2.10g $100g^{-1}$ methionine and lysine, respectively (Bulbul et al., 2013). Similarly, fish such as the Atlantic salmon showed that excessive dietary Met can negatively affect fish feed intake, growth performance, and survival due to toxic effects (Espe et al., 2008). Such response are possibly due to leaching, amino acid imbalances and the faster absorption and catabolism of AA in the crystalline form than that of the intact protein form (Sveier et al., 2001; El Haroun and Bureau, 2007; Hauler et al., 2007; Dabrowski et al., 2010).

4.2 Shrimp composition and retention

Shrimp proximate composition did not show significant differences for either experimental trial (Tables 6 and 7). Significant differences were only observed in EAAs such as arginine, histidine, phenylalanine, and taurine having significant differences for Trial 1, and threonine and taurine for Trial 2. Variations in shrimp whole body amino acid composition may be attributed to

differences in amino acid composition of the diets. CPC contains high levels of arginine, tryptophan, threonine, and several dispensable amino acids (Khalaji et al., 2016), hence as the diets are blended this is a shift in dietary amino acids. The increase in EAAs associated with increasing Met levels suggests a dose response to methionine.

In the present study, a general increase in protein retention as METH from IP increased, this would indicate improvements in protein deposition likely due to a better balance of EAA. In this research, lysine levels of the replete IP diet were lower than that of the basal diet, albeit well above the requirement. Hence, as the diets were blended to increase METH from IP (primarily from CPC), lysine decreased resulting in improved lysine retention. In contrast to lysine, a decreasing trend of methionine retention was observed as dietary methionine inclusion increased, for both crystalline Met sources DL-Met and Met-Met and for IP diets as well, in both trials (Table 8), which was also observed in previous studies (Millamena et al., 1996; Lin et al., 2015; Façanha et al., 2018). The response decreased to around 0.6 % methionine and then leveled out which also supports a response to a deficiency and then a replete diet. Confirming a response to methionine levels of the diet.

The current study is similar to a study in pacu (*Piaractus mesopotamicus*) that evaluated nitrogen retention and excretion, and showed that dietary methionine demonstrated satisfactory growth (Abimorad et al., 2009). In another study on rainbow trout, methionine supplementation, as methionine hydroxy analogue (MHA) at 1.65g per kg of the diet, demonstrated better weight gain, FCR, apparent crude protein retention, and phosphorus in trout, as compared to trout fed diets without MHA (Cheng et al., 2003).

4.4 Methionine Requirement

The most common method to determine a requirement is to evaluate various models describing dose response (Robbins, 1986). For this work, several models were evaluated but only a single slope method was found suitable for trial data sets from both trials. The current study estimates the dietary methionine requirement for maximum growth to be 0.6659% of dry diet, corresponding to 1.85% of dietary protein for Trial 1 (Figure 1) and 0.5588% of dry diet, corresponding to 1.55% of dietary protein for Trial 2 (Figure 3) based on a broken-line regression analysis on weight gain (%). Similarly, for TGC, the optimum dietary methionine level for maximum growth was estimated to be 0.6559% of dry diet, corresponding to 1.82% of dietary protein for Trial 1 (Figure 2) and at 0.6053% of dry diet, corresponding to 1.68% of dietary protein for Trial 2 (Figure 4). There are several studies in the literature that have reported on dietary requirements that were both lower and higher than our determined values. For example, the results of the present study are lower than the Met requirement of 2.28% of dietary protein for smaller-sized shrimp and 1.76% for larger-sized shrimp (Lin et al., 2015), 2.25% of the protein (Wang et al., 2019) and 2.52% protein (Huai et al., 2009) as well are the 2.4% of dietary protein reported for black tiger shrimp, *P. monodon*, (Millamena et al., 1996). All of which are all relatively higher than the estimates of the current study (1.68-1.82% protein).

Although no experiment is perfect and often times methods are not always clear, it is interesting to note that quite often there are possible issues with analysis. For example, mean data is sometimes used for regression analysis, there is limited replication and/or treatment levels and in some cases graphical representation does not match the data as described in the paper. This is further complicated by requirements being reported as that of a methionine when, in this author's view, it would be a TSAA requirement. For example, (Wang et al., 2019) reported a methionine requirement of 0.85% of the diet. In the diet closest to the requirement (MM 0.25) the methionine

content was 0.84% and cystine 0.39%. This means cystine represents 31% of the TSAA levels, which would indicate that cysteine is limiting and that the "methionine" requirement should be presented as TSAA of about 1.24% diet or 3.2% protein.

Discrepancies and inconsistencies in studies attempting to determine Met requirement may be attributed to differences in experimental design, shrimp species and age, CAA form and source, as well as Met processing (coating, encapsulation, microencapsulation or polymerization), which have all been used to reduce leaching losses and absorption rate (Villamar and Langdon, 1993; Guo et al., 2020). Aside from this, different studies do not consider Cys levels as part of the TSAA composition in the diet. Since Met is required for cysteine synthesis as its metabolic precursor, and any substitution by Cys for dietary Met requirement can only be via inhibition of the sulfur amino acid pathway that leads to synthesis of the transsulfuration metabolites, including cysteine itself (Ball et al., 2006), it is important to consider Cys levels due to its sparing effect on Met. For instance, in a study on Nile tilapia, cystine could replace up to approximately 47% of TSAA requirement on an equimolar sulfur basis without hampering growth performance in practical diets of juvenile Nile tilapia (He et al., 2016). Hence, for the current study, Cys was formulated to be in excess to be able to account for the mentioned sparing effect of Cys on Met, and to make sure that it is indeed Met requirement that is determined and not TSAA.

Various studies have estimated shrimp methionine requirement differently. When using practical diets with 34% to 40% crude protein under clear water conditions for shrimp ranging from 0.55 ± 0.01 to 9.77 ± 0.08 g body weight, respectively, methionine requirements ranged from 0.66% to 0.91% of the diet (1.94% to 2.28% or crude protein) (Lin et al., 2015). In another study, dietary Met requirement for *L. vannamei* was estimated to be 0.74% of the diet (3.70% of crude protein) using semi-purified diets with 20% crude protein content (Fox et al., 2010). According to

NRC (2011), dietary methionine requirements for shrimp species range from 2-3% of dietary protein but are not reported for the pacific white shrimp as there was not sufficient data at the time of writting. This wide variability between different aquaculture species may be due to differences in size, age, experimental conditions, such as water temperature, salinity, feeding regime, feed allowance, stock density, flow rate, production system as well as the use of different ingredients for basal diets such as commercial or purified ingredients (Lin et al., 2015).

5. Conclusion

In summary, the results of this study demonstrated the response of white shrimp when fed with Met supplemented diets through the use of DL-Met and Met-Met as well as the intact protein CPC. Growth performance such as TGC significantly improved as Met supplementation with CPC increased, as verified by two experimental trials. Shrimp fed Met-Met supplemented diets performed better than DL-Met fed shrimp, specifically for weight gain (%), FCR, and TGC. Nutrient retention values for protein and lysine significantly increased as Met supplementation increased, while Met retention significantly decreased as Met supplementation increased. The utilization of CPC as an intact protein source showed a dose response in all growth performance parameters and nutrient retention values and demonstrated significantly better results than CAA supplementation.

The optimal dietary methionine requirement of *L. vannamei*, estimated by a one-slope broken-line regression analysis model based on weight gain (%) ranged between was 0.5588 to 0.6659% of the dry diet (corresponding to 1.55-1.84% of dietary protein on a dry-weight basis), respectively. For TGC (thermal-unit growth coefficient), the optimal dietary methionine requirement ranged between 0.6053 to 0.6559% of the dry diet (corresponding to 1.68-1.82% of

dietary protein on a dry-weight basis), respectively. Such findings are crucial in formulating cost-effective practical diets and utilizing intact proteins or purified CAAs for juvenile *L. vannamei*.

Table 1: Formulation of experimental diets used to determine the dietary methionine requirement of juvenile Pacific white shrimp, *Litopenaeus vannamei*.

	Trial 1			Trial 2		
Ingredient (% as is)	Basal	Basal + DL-Met	Intact Protein	Basal	Basal + Met-Met	Intact Protein
Soybean meal [1]	0.00	0.00	27.3	13.80	0.00	22.0
Fish meal [2]	5.00	5.00	5.00	5.00	13.05	5.00
Corn protein concentrate [3]	0.00	0.00	20.00	0.00	0.00	20.00
Lentil meal [4]	49.20	49.20	0.00	35.00	35.00	0.00
Whole wheat [5]	33.80	33.80	33.80	28.00	28.00	28.00
Corn starch [6]	0.00	0.00	1.69	2.20	2.60	8.10
Fish oil [7]	4.39	4.39	5.66	4.45	4.45	5.35
Krill meal[8]	0.00	0.00	0.00	5.00	5.00	5.00
Soy lecithin [9]	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol [6]	0.15	0.15	0.15	0.15	0.15	0.15
CaP-dibasic [6]	2.80	2.80	2.80	2.80	2.80	2.80
Trace mineral premix [10]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix [11]	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride [6]	0.20	0.20	0.20	0.20	0.20	0.20
Stay C [12]	0.10	0.10	0.10	0.10	0.10	0.10
Cysteine	0.00	0.53	0.00	0.00	0.00	0.00
Glutamic acid	0.53	0.00	0.00	0.00	0.00	0.00
Glycine	0.53	0.00	0.00	0.00	0.00	0.00

DL-Methionine [13]	0.00	0.53	0.00	0.00	0.00	0.00
Met-Met [14]	0.00	0.00	0.00	0.00	0.35	0.00

[1] Solvent Extracted Soybean Meal, De-hulled solvent-extracted soybean meal, Bunge Limited, Decatur, AL, USA.
[2] Omega Protein Inc, Houston, Texas, USA.
[3] Empyreal 75 TM Cargill Corn Milling, Cargill Inc., Blair, Nebraska, USA.
[4] AGT Food and Ingredients, Inc. Saskatchewan, Canada.
[5] Bobs Red Mill, Milwaukie, OR, USA.
[6] MP Biomedicals Inc., Solon, OH, USA.
[7] Omega Protein Inc., Reedville, Virginia, USA.
[8] QrillTM Aqua, Aker Biomarine AS, Oslo, Norway
[9] The Solae Company, St. Louis, MO, USA.
[10] Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[11] Vitamin premix (g/kg premix): Thiamin HCL, 4.95; Riboflavin, 3.83; Pyridoxine HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[12] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, New Jersey, USA.
[13] Feed Grade 99% Min.
[14] AQUAVI®, Evonik Nutrition and Care GmbH, Hanau, Germany.

Table 2: Proximate composition (% as is) and amino acid profile (% as is) of experimental diets used in Trial 1.

		DL-Met		Intact Protein						
Composition[1]	Basal	B70:DM30	DM100	B90:C10	B80:C20	B70:C30	B60:C40	B40:C60	B20:C80	B0:C100
Crude protein	35.99	35.21	35.55	35.28	37.14	37.79	36.81	37.47	38.30	39.13
Moisture	7.73	8.57	7.07	7.95	7.38	7.61	6.93	6.88	6.65	6.74
Crude fat	5.74	5.15	6.15	7.35	4.58	4.49	7.46	6.94	6.62	6.26
Crude fiber	2.08	2.84	2.59	2.96	2.88	2.78	3.12	3.09	3.11	3.48
Ash	6.23	5.88	6.15	6.08	6.07	6.09	5.93	6.01	6.20	6.16
Alanine	1.42	1.38	1.44	1.47	1.68	1.78	1.79	1.98	2.16	2.43
Arginine	2.52	2.45	2.56	2.41	2.43	2.38	2.24	2.12	2.03	1.92
Aspartic Acid	3.33	3.24	3.37	3.19	3.31	3.35	3.12	3.14	3.09	3.00
Cysteine	0.40	0.54	0.91	0.54	0.46	0.48	0.48	0.55	0.62	0.62
Glutamic Acid	6.70	6.37	6.18	6.78	7.04	7.14	7.12	7.42	7.71	8.09
Glycine	1.94	1.71	1.48	1.88	1.90	1.89	1.71	1.62	1.54	1.48
Histidine	0.84	0.80	0.85	0.82	0.86	0.87	0.84	0.85	0.87	0.89
Hydroxylysine	0.00	0.00	0.00	0	0.00	0.01	0.00	0.00	0.00	0.00
Hydroxyproline	0.11	0.10	0.10	0.09	0.09	0.11	0.10	0.10	0.07	0.08
Isoleucine	1.55	1.50	1.55	1.49	1.59	1.59	1.57	1.62	1.64	1.70
Lanthionine	0.05	0.05	0.05	0.03	0.05	0.05	0.06	0.06	0.06	0.06
Leucine	2.51	2.43	2.52	2.61	2.96	3.11	3.19	3.53	3.86	4.34
Lysine	2.21	2.06	2.20	2.05	2.05	2.02	1.87	1.74	1.66	1.53
Methionine	0.38	0.51	0.87	0.48	0.48	0.52	0.52	0.63	0.72	0.75
Ornithine	0.05	0.05	0.05	0.07	0.05	0.05	0.04	0.03	0.03	0.02
Phenylalanine	1.75	1.69	1.76	1.75	1.86	1.89	1.87	1.95	2.02	2.14
Proline	1.73	1.68	1.73	1.8	2.00	2.10	2.15	2.48	2.67	2.91

Serine	1.42	1.38	1.43	1.52	1.50	1.55	1.50	1.54	1.54	1.61
Taurine	0.23	0.20	0.20	0.2	0.23	0.22	0.21	0.21	0.24	0.21
Threonine	1.18	1.14	1.19	1.17	1.24	1.27	1.21	1.25	1.28	1.31
Tryptophan	0.32	0.29	0.30	0.32	0.32	0.33	0.34	0.36	0.36	0.36
Tyrosine	1.05	1.00	1.04	1.1	1.14	1.17	1.22	1.31	1.40	1.53
Valine	1.70	1.65	1.71	1.62	1.76	1.75	1.71	1.75	1.79	1.84

[1] Diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 3: Proximate composition (% as is) and amino acid profile (% as is) of experimental diets used in Trial 2.

		Met-Met		Intact Protein						
Composition[1]	Basal	B70:MM30	MM100	B90:C10	B80:C20	B70:C30	B60:C40	B40:C60	B20:C80	B0:C100
Crude protein	35.22	34.32	34.76	34.38	34.29	34.96	35.09	34.94	35.89	37.01
Moisture	6.97	8.92	8.38	8.43	9.01	7.70	7.58	9.26	7.65	6.25
Crude fat	7.92	8.24	7.94	8.33	8.09	8.12	8.93	8.63	9.07	9.64
Crude fiber	11.28	7.51	9.10	12.74	9.02	7.09	12.1	12.14	10.72	16.04
Ash	7.34	7.05	7.17	7.01	6.74	6.92	6.79	6.51	6.38	6.37
Alanine	1.51	1.47	1.47	1.52	1.61	1.76	1.81	1.92	2.13	2.37
Arginine	2.51	2.44	2.45	2.36	2.32	2.30	2.22	2.03	1.93	1.83
Aspartic Acid	3.49	3.40	3.38	3.29	3.30	3.35	3.24	3.04	2.99	2.98
Cysteine	0.42	0.42	0.39	0.42	0.44	0.47	0.48	0.49	0.54	0.63
Glutamic Acid	6.12	5.98	5.98	6.04	6.20	6.51	6.60	6.70	7.02	7.43
Glycine	1.52	1.47	1.49	1.45	1.46	1.48	1.46	1.41	1.45	1.50
Histidine	0.85	0.83	0.82	0.82	0.82	0.84	0.84	0.81	0.82	0.84
Hydroxylysine	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Hydroxyproline	0.10	0.09	0.10	0.09	0.08	0.07	0.09	0.07	0.06	0.10
Isoleucine	1.60	1.58	1.57	1.57	1.59	1.65	1.65	1.61	1.63	1.71
Lanthionine	0.05	0.06	0.05	0.06	0.06	0.06	0.06	0.06	0.07	0.07
Leucine	2.56	2.51	2.50	2.60	2.77	3.04	3.14	3.34	3.69	4.11
Lysine	2.29	2.23	2.24	2.15	2.10	2.11	2.04	1.83	1.74	1.67
Methionine	0.48	0.54	0.85	0.50	0.53	0.58	0.61	0.65	0.72	0.84
Ornithine	0.05	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.03	0.06
Phenylalanine	1.73	1.67	1.69	1.69	1.73	1.82	1.83	1.83	1.91	2.02
Proline	1.72	1.71	1.73	1.80	1.91	2.05	2.10	2.26	2.50	2.73

Serine	1.43	1.40	1.42	1.36	1.37	1.40	1.40	1.38	1.43	1.51
Taurine	0.23	0.22	0.23	0.21	0.21	0.21	0.21	0.21	0.21	0.23
Threonine	1.25	1.22	1.21	1.18	1.21	1.25	1.23	1.20	1.24	1.29
Tryptophan	0.33	0.33	0.33	0.32	0.31	0.33	0.33	0.31	0.32	0.35
Tyrosine	1.12	1.08	1.09	1.09	1.13	1.23	1.24	1.28	1.37	1.47
Valine	1.71	1.67	1.66	1.67	1.70	1.77	1.75	1.70	1.74	1.80

[1] Diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 4: Response of juvenile Pacific white shrimp, *Litopenaeus vannamei* (mean initial weight = 0.45 ± 0.002g) fed diets containing graded levels of methionine from DL-methionine (DM) or intact protein (C) over a 54-day feeding trial (Trial 1). Values represent the mean of six replicates. One-way analysis of variance and Tukey post-hoc test was used to determine significant differences ($p < 0.05$) among treatments (n = 6), represented by values with different letters.

	Met[1]	Cys[1]	Biomass (g)	Final weight (g)	Weight Gain (g)	Weight Gain (%)	FCR[2]	Survival (%)	TGC[3]
Basal	0.38	0.40	53.15 ^{bcd}	3.92 ^{c}	3.47 ^{a}	775.19 ^{d}	2.54 ^{bc}	91.11 ^{a}	0.0526 ^{cd}
B70:DM30	0.51	0.54	42.85 ^{d}	3.08 ^{d}	2.63 ^{d}	595.25 ^{e}	3.34 ^{a}	93.33 ^{a}	0.0449 ^{e}
DM100	0.87	0.91	54.55 ^{bc}	4.09 ^{bc}	3.64 ^{bc}	809.76 ^{cd}	2.41 ^{bcd}	88.89 ^{ab}	0.0541 ^{cd}
B90:C10	0.35	0.37	49.15 ^{cd}	3.84 ^{c}	3.39 ^{c}	752.01 ^{de}	2.59 ^{b}	85.56 ^{ab}	0.0519 ^{d}
B80:C20	0.48	0.46	55.05 ^{bc}	4.29 ^{bc}	3.84 ^{bc}	855.62 ^{cd}	2.28 ^{bcd}	85.56 ^{ab}	0.0558 ^{bcd}
B70:C30	0.51	0.48	57.43 ^{abc}	4.81 ^{ab}	4.36 ^{ab}	973.26 ^{abc}	2.02 ^{def}	80.00 ^{ab}	0.0599 ^{ab}
B60:C40	0.54	0.50	57.03 ^{bc}	4.51 ^{bc}	4.07 ^{bc}	919.75 ^{bcd}	2.15 ^{cde}	84.45 ^{ab}	0.0578 ^{bc}
B40:C60	0.63	0.55	68.95 ^{a}	5.37 ^{a}	4.91 ^{a}	1069.84 ^{ab}	1.78 ^{ef}	85.56 ^{ab}	0.0636 ^{a}
B20:C80	0.72	0.62	59.35 ^{abc}	5.40 ^{a}	4.94 ^{a}	1089.70 ^{a}	1.80 ^{ef}	73.33 ^{b}	0.0639 ^{a}
B0:C100	0.75	0.62	64.30 ^{ab}	5.54 ^{a}	5.09 ^{a}	1122.77 ^{a}	1.73 ^{f}	77.78 ^{ab}	0.0650 ^{a}
PSE[4]			1.15	0.11	0.11	0.24	0.07	1.23	0.0009
P-value			0.0001	0.0001	0.0001	0.0001	0.0001	0.0031	0.0001

[1]Met and Cys values were from analysis by University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).
[2]FCR = Feed conversion ratio = feed offered / (final weight-initial weight).
[3]TGC = Thermal-unit growth coefficient.

[4]PSE = Pooled standard error.

Table 5: Response of juvenile Pacific white shrimp, *Litopenaeus vannamei* (mean initial weight = 0.23 ± 0.0001g) fed diets containing graded levels of methionine from Met-Met (MM) or intact protein (C) over a 42-day feeding trial (Trial 2). Values represent mean of six replicates. One-way analysis of variance and Tukey post-hoc test was used to determine significant differences ($p < 0.05$) among treatments (n = 6), represented by values with different letters.

	Met[1]	Cys[1]	Biomass (g)	Final weight (g)	Weight Gain (g)	Weight Gain (%)	FCR[2]	Survival (%)	TGC[3]
Basal	0.48	0.42	64.22	4.84^{ab}	4.61^{ab}	2028.07	1.78	88.89	0.0811
B70:MM30	0.54	0.42	58.43	4.58^{b}	4.35^{b}	1927.75	1.91	84.45	0.0791
MM100	0.85	0.39	60.93	4.90^{ab}	4.67^{ab}	2047.92	1.80	83.33	0.0816
B90:C10	0.50	0.42	65.26	4.94^{ab}	4.71^{ab}	2066.67	1.76	87.78	0.0820
B80:C20	0.53	0.44	64.45	5.06^{ab}	4.84^{ab}	2152.21	1.73	85.56	0.0833
B70:C30	0.58	0.47	61.89	5.11^{ab}	4.89^{ab}	2247.52	1.70	81.11	0.0843
B60:C40	0.61	0.48	62.75	4.90^{ab}	4.67^{ab}	2063.39	1.81	85.56	0.0816
B40:C60	0.65	0.49	64.72	5.18^{ab}	4.96^{ab}	2236.14	1.68	83.34	0.0846
B20:C80	0.72	0.54	61.71	5.54^{a}	5.30^{a}	2285.66	1.55	74.44	0.0868
B0:C100	0.84	0.63	71.37	5.54^{a}	5.31^{a}	2338.39	1.55	85.56	0.0871
PSE[4]			1.17	0.07	0.07	32.80	0.03	1.26	0.0006
p-value			0.5861	0.0273	0.027	0.0814	0.0504	0.4315	0.0585

[1]Met and Cys values were from analysis by University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).
[2]FCR = Feed conversion ratio = feed offered / (final weight-initial weight).
[3]TGC = Thermal-unit growth coefficient.
[4]PSE = Pooled standard error.

Table 6: Proximate composition (% as is) and amino acid profile (% as is) of whole-body shrimp fed different methionine sources for Trial 1. Values represent mean of six replicates.

	DL-Met			Intact Protein						
Composition[1]	Basal	B70:DM30	DM100	B90:C10	B80:C20	B70:C30	B60:C40	B40:C60	B20:C80	B0:C100
Crude protein	74.46	75.69	74.78	75.10	75.83	76.25	74.49	75.13	74.77	74.61
Moisture	4.76	4.37	4.33	4.31	4.04	3.96	4.77	4.25	4.48	4.55
Crude fat	5.43	3.85	5.69	4.66	4.45	4.14	6.42	6.22	6.45	6.54
Ash	9.98	10.36	9.88	10.18	9.97	9.93	9.44	9.18	9.15	9.23
Alanine	4.37^{ab}	4.28^{b}	4.28^{b}	4.36^{ab}	4.39^{ab}	4.41^{ab}	4.44^{ab}	4.43^{ab}	4.40^{ab}	4.49^{a}
Arginine	5.41^{ab}	5.48^{ab}	5.39^{ab}	5.44^{ab}	5.38^{ab}	5.52^{a}	5.31^{ab}	5.40^{ab}	5.31^{ab}	5.29^{b}
Aspartic Acid	6.74	6.88	6.69	6.74	6.86	6.87	6.74	6.78	6.79	6.85
Cysteine	0.66	0.67	0.66	0.65	0.67	0.68	0.66	0.66	0.66	0.68
Glutamic Acid	10.50	10.55	10.25	10.48	10.55	10.66	10.39	10.57	10.50	10.53
Glycine	5.18^{b}	5.72^{a}	5.04^{bc}	5.15^{bc}	5.12^{bc}	5.16^{bc}	5.06^{bc}	4.93^{bc}	4.84^{c}	4.94^{bc}
Histidine	1.63^{ab}	1.66^{a}	1.58^{b}	1.60^{ab}	1.64^{ab}	1.62^{ab}	1.58^{b}	1.60^{ab}	1.59^{b}	1.59^{b}
Hydroxylysine	0.11	0.11	0.11	0.11	0.11	0.12	0.11	0.11	0.11	0.10
Hydroxyproline	0.31	0.32	0.29	0.33	0.37	0.30	0.36	0.30	0.27	0.26
Isoleucine	3.01	3.05	2.96	2.97	3.04	3.05	3.01	3.01	3.01	3.05
Lanthionine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Leucine	4.88	4.95	4.83	4.86	4.94	4.99	4.88	4.92	4.92	4.95
Lysine	5.13	5.14	5.03	5.08	5.14	5.19	5.06	5.10	5.09	5.10
Methionine	1.48	1.50	1.47	1.46	1.51	1.52	1.50	1.49	1.50	1.52
Ornithine	0.24	0.24	0.25	0.25	0.24	0.26	0.24	0.25	0.25	0.24
Phenylalanine	3.23^{ab}	3.31^{ab}	3.21^{b}	3.24^{ab}	3.27^{ab}	3.33^{a}	3.22^{ab}	3.27^{ab}	3.26^{ab}	3.25^{ab}
Proline	3.61^{abc}	3.44^{c}	3.59^{abc}	3.52^{bc}	3.69^{abc}	3.62^{abc}	3.64^{abc}	3.71^{ab}	3.80^{a}	3.77^{ab}
Serine	2.43	2.52	2.40	2.47	2.48	2.51	2.43	2.49	2.45	2.47
Taurine	0.43^{e}	0.34^{f}	0.55^{bcd}	0.51^{d}	0.52^{d}	0.56^{abcd}	0.53^{cd}	0.59^{ab}	0.61^{a}	0.58^{abc}
Threonine	2.64	2.69	2.63	2.63	2.67	2.69	2.62	2.64	2.64	2.64
Tryptophan	0.84	0.87	0.85	0.83	0.86	0.88	0.85	0.86	0.84	0.86
Tyrosine	2.48	2.49	2.49	2.51	2.53	2.58	2.50	2.55	2.52	2.48

Valine	3.87	3.94	3.84	3.95	3.96	4.01	3.84	3.95	3.90	3.91

[1]Shrimp whole body samples were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Note: One-way analysis of variance and Tukey post-hoc test was used to determine significant differences ($p < 0.05$) among treatments (n = 6), represented by values with different letters.

Table 7: Proximate composition (% as is) and amino acid profile (% as is) of whole-body shrimp fed different methionine sources for Trial 2. Values represent the mean of six replicates.

		Met-Met		Intact Protein						
Composition[1]	Basal	B70:MM30	MM100	B90:C10	B80:C20	B70:C30	B60:C40	B40:C60	B20:C80	B0:C100
Crude protein	71.61	70.93	70.33	73.03	71.25	71.58	69.42	69.41	69.27	67.92
Moisture	3.67	3.68	3.43	3.27	3.78	3.63	3.53	3.45	3.68	3.58
Crude fat	6.45	7.03	7.23	6.64	6.65	7.37	7.77	7.82	8.57	9.68
Ash	11.65	11.29	11.68	12.01	11.55	11.07	11.69	11.25	11.25	11.34
Alanine	4.57	4.78	4.56	4.66	4.72	4.64	4.45	4.46	4.43	4.53
Arginine	5.02	5.26	5.05	5.01	5.30	5.15	4.91	4.78	4.69	4.66
Aspartic Acid	6.24	6.50	6.35	6.28	6.63	6.41	6.30	6.00	6.09	6.17
Cysteine	0.56^{ab}	0.60^{ab}	0.59^{ab}	0.53^{b}	0.62^{a}	0.59^{ab}	0.58^{ab}	0.54^{b}	0.57^{ab}	0.57^{ab}
Glutamic Acid	9.86	10.36	9.87	10.19	10.38	10.08	9.81	9.36	9.32	9.64
Glycine	4.30^{ab}	4.52^{a}	4.40^{ab}	4.36^{ab}	4.46^{ab}	4.28^{ab}	4.21^{ab}	4.20^{ab}	4.10^{b}	4.1^{b}
Histidine	1.32	1.38	1.33	1.30	1.40	1.36	1.31	1.27	1.32	1.30
Hydroxylysine	0.14	0.09	0.15	0.16	0.13	0.13	0.15	0.15	0.12	0.12
Hydroxyproline	0.18	0.23	0.18	0.15	0.19	0.20	0.19	0.18	0.21	0.19
Isoleucine	2.88	3.02	2.93	2.92	3.05	2.96	2.89	2.76	2.79	2.86
Lanthionine	0.07	0.07	0.05	0.07	0.07	0.07	0.06	0.07	0.09	0.10
Leucine	4.69	4.93	4.76	4.76	5.01	4.81	4.73	4.48	4.53	4.67
Lysine	4.80	5.15	4.88	4.81	5.21	4.95	4.77	4.51	4.55	4.64
Methionine	1.37	1.45	1.44	1.42	1.48	1.42	1.42	1.33	1.35	1.37
Ornithine	0.35	0.37	0.36	0.35	0.35	0.34	0.33	0.33	0.33	0.32
Phenylalanine	2.95	3.00	3.06	3.01	3.11	2.98	2.96	2.91	2.86	2.95
Proline	3.73	3.88	3.70	3.56	3.87	3.83	3.76	3.72	3.72	3.84
Serine	2.17	2.24	2.17	2.18	2.29	2.17	2.20	2.10	2.08	2.12

Taurine	0.32^{d}	0.34^{cd}	0.41^{abc}	0.31^{d}	0.34^{bcd}	0.35^{bcd}	0.38^{abcd}	0.42^{ab}	0.45^{a}	0.44^{a}
Threonine	2.55^{ab}	2.63^{ab}	2.56^{ab}	2.56^{ab}	2.67^{a}	2.58^{ab}	2.56^{ab}	2.43^{b}	2.45^{ab}	2.49^{ab}
Tryptophan	0.64	0.65	0.64	0.61	0.64	0.62	0.64	0.63	0.63	0.64
Tyrosine	2.25	2.34	2.24	2.27	2.34	2.32	2.26	2.27	2.27	2.27
Valine	3.23	3.44	3.27	3.24	3.41	3.36	3.18	3.20	3.27	3.26

[1]Shrimp whole body samples were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Note: One-way analysis of variance and Tukey post-hoc test was used to determine significant differences ($p < 0.05$) among treatments (n = 6), represented by values with different letters.

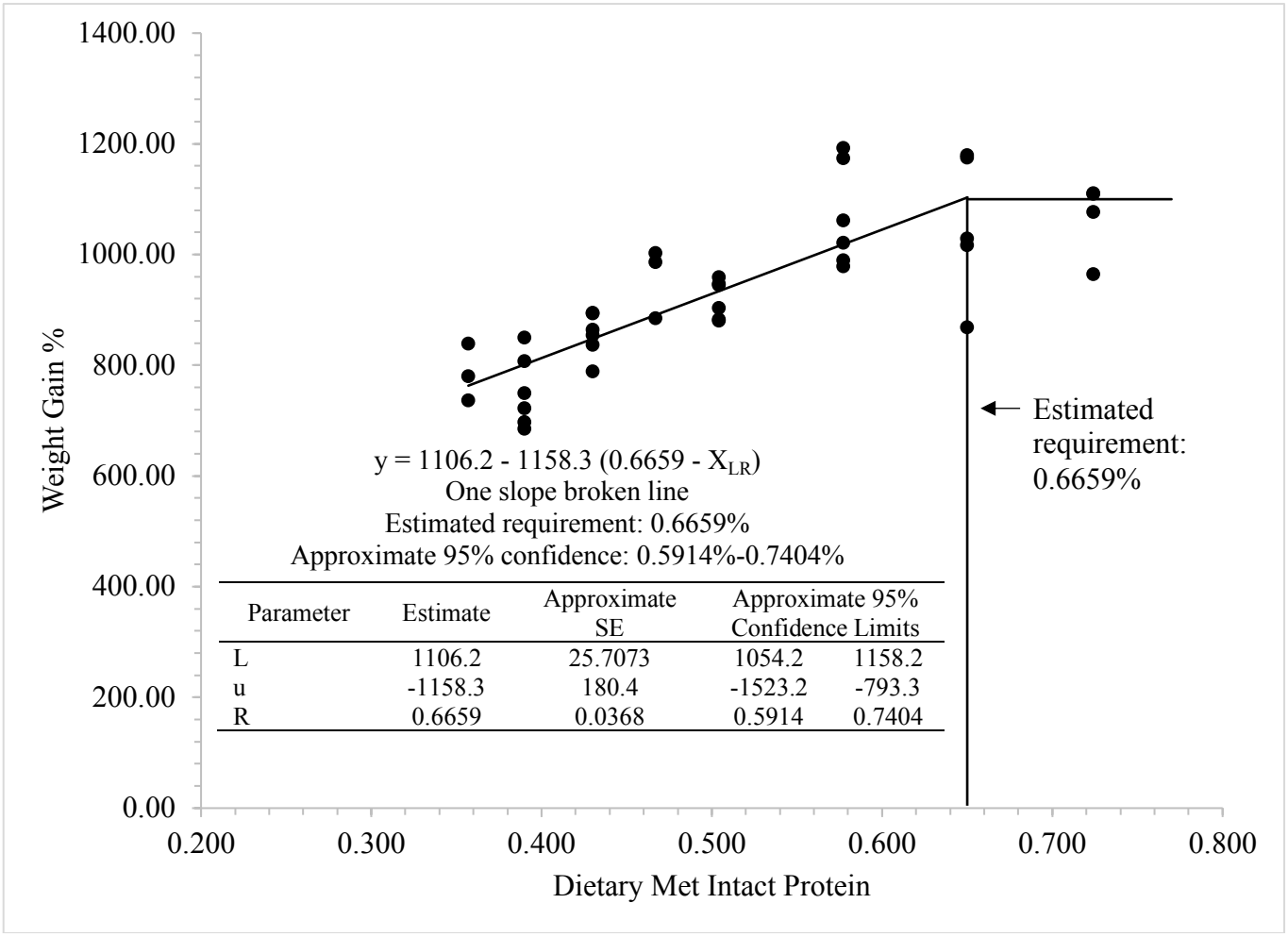
Table 8: Amino acid retention values of juvenile Pacific white shrimp, *Litopenaeus vannamei* fed different methionine sources, for Trials 1 and 2. Values represent mean of six replicates. One-way analysis of variance and Tukey post-hoc test were used to determine significant differences ($p < 0.05$) among treatments (n = 6), represented by values with different letters.

	Trial l				
	Met	Cys	Protein Retention	Methionine Retention	Lysine Retention
Basal	0.38	0.40	18.68 ^{ab}	35.40^{a}	21.03^{cde}
B70:DM30	0.51	0.54	12.72^{b}	17.70^{cd}	14.83^{e}
DM100	0.87	0.91	19.04^{ab}	15.39^{d}	20.75^{de}
B90:C10	0.35	0.37	21.22^{ab}	36.87^{a}	26.21^{bcde}
B80:C20	0.48	0.46	18.38^{ab}	28.48^{abc}	22.60^{cde}
B70:C30	0.51	0.48	19.39^{ab}	28.31^{abc}	24.66^{bcde}
B60:C40	0.54	0.50	21.16^{ab}	30.39^{ab}	28.38^{bcd}
B40:C60	0.63	0.55	22.63^{a}	26.73^{abc}	33.04^{abc}
B20:C80	0.72	0.62	22.11^{a}	23.74^{bcd}	34.93^{ab}
B0:C100	0.75	0.62	24.82^{a}	26.49^{abcd}	43.47^{a}
p-value			0.006	<0.0001	<0.0001
	Trial 2				
	Met	Cys	Protein Retention	Methionine Retention	Lysine Retention
Basal	0.48	0.42	27.18^{ab}	37.78^{a}	27.96^{cde}
B70:M30	0.54	0.42	26.27^{b}	34.00^{cd}	29.48^{e}
M100	0.85	0.39	27.58^{ab}	23.03^{d}	29.80^{de}
B90:C10	0.50	0.42	28.69^{ab}	38.01^{a}	30.13^{bcde}
B80:C20	0.53	0.44	29.58^{ab}	39.55^{abc}	35.37^{cde}
B70:C30	0.58	0.47	29.46^{ab}	35.03^{abc}	33.79^{bcde}

B60:C40	0.61	0.48	26.80^{ab}	31.33^{ab}	31.66^{bcd}
B40:C60	0.65	0.49	29.23^{a}	29.89^{abc}	36.22^{abc}
B20:C80	0.72	0.54	30.61^{a}	29.55^{bcd}	41.45^{ab}
B0:C100	0.84	0.63	30.64^{a}	27.11^{abcd}	46.44^{a}
p-value			0.0087	<0.0001	<0.0001

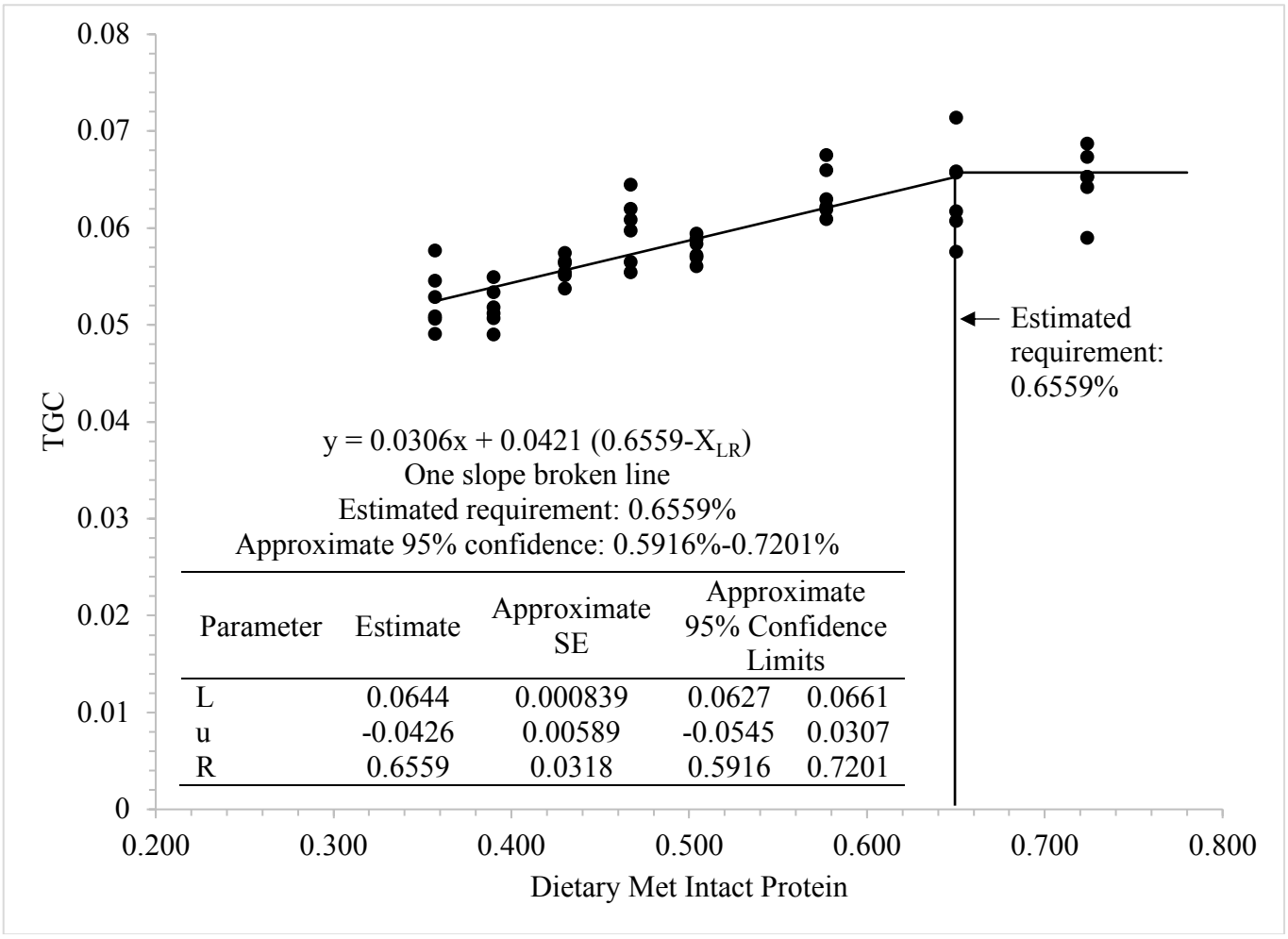
[1]Met and Cys values were from analysis by University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Figure 1: Weight gain (%) of juvenile Pacific white shrimp, *Litopenaeus vannamei* fed intact protein for Trial 1. The one slope line model estimated that optimal weight gain (%) was obtained with a dietary methionine requirement of 0.67%, or 1.85% of protein, based on dry weight.



Parameter	Estimate	Approximate SE	Approximate 95% Confidence Limits	
L	1106.2	25.7073	1054.2	1158.2
u	-1158.3	180.4	-1523.2	-793.3
R	0.6659	0.0368	0.5914	0.7404

Figure 2: Thermal growth coefficient of juvenile Pacific white shrimp, *Litopenaeus vannamei* fed intact protein for Trial 1. The one slope line model estimated that optimal TGC was obtained with a dietary methionine requirement of 0.66%, or 1.82% of protein, based on dry weight.



Parameter	Estimate	Approximate SE	Approximate 95% Confidence Limits	
L	0.0644	0.000839	0.0627	0.0661
u	-0.0426	0.00589	-0.0545	0.0307
R	0.6559	0.0318	0.5916	0.7201

Figure 3: Weight gain (%) of juvenile Pacific white shrimp, *Litopenaeus vannamei* fed intact protein for Trial 2. The one slope line model estimated that optimal weight gain % was obtained with a dietary methionine requirement of 0.56% or 1.56% of protein, based on dry weight.

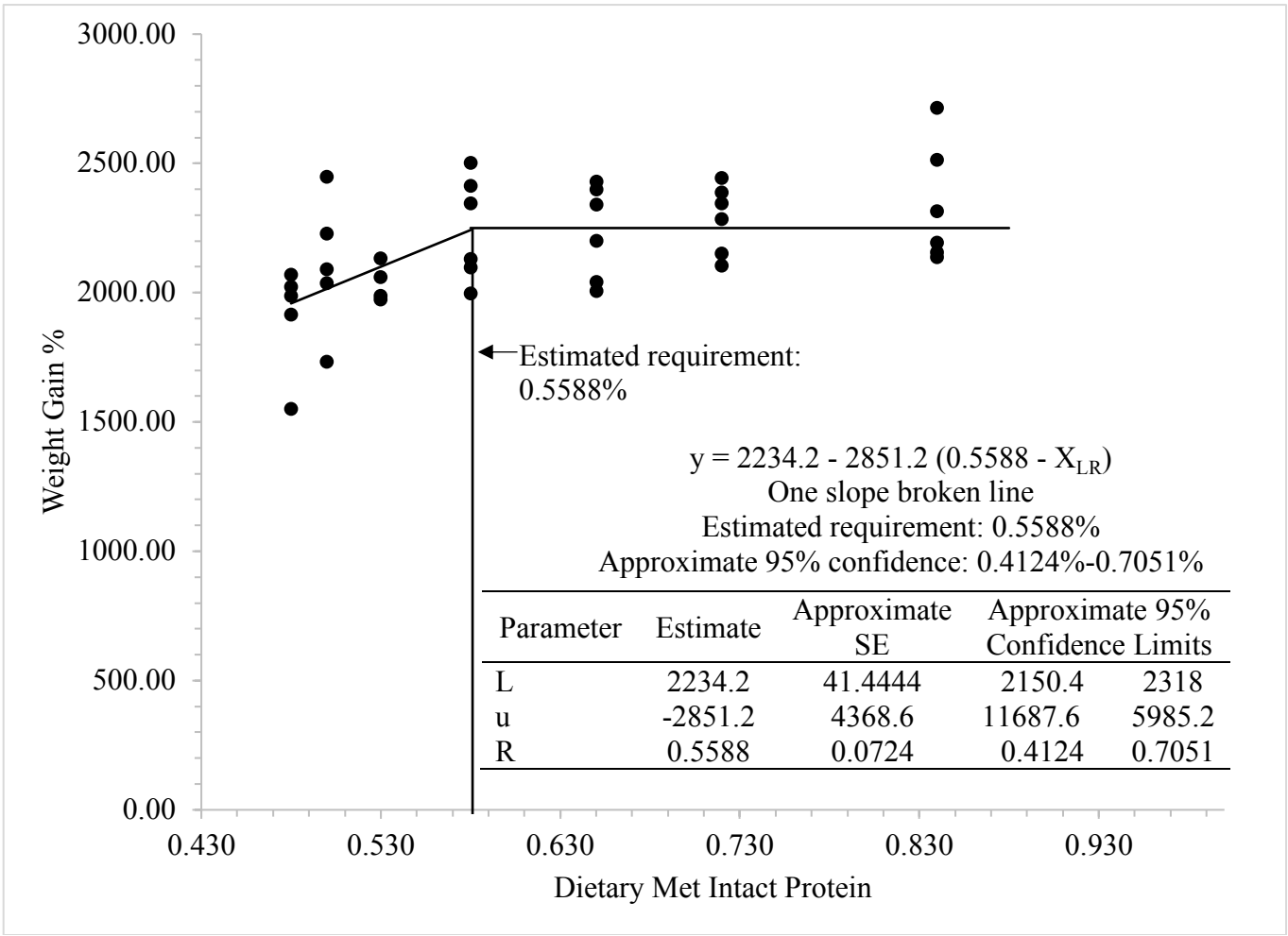
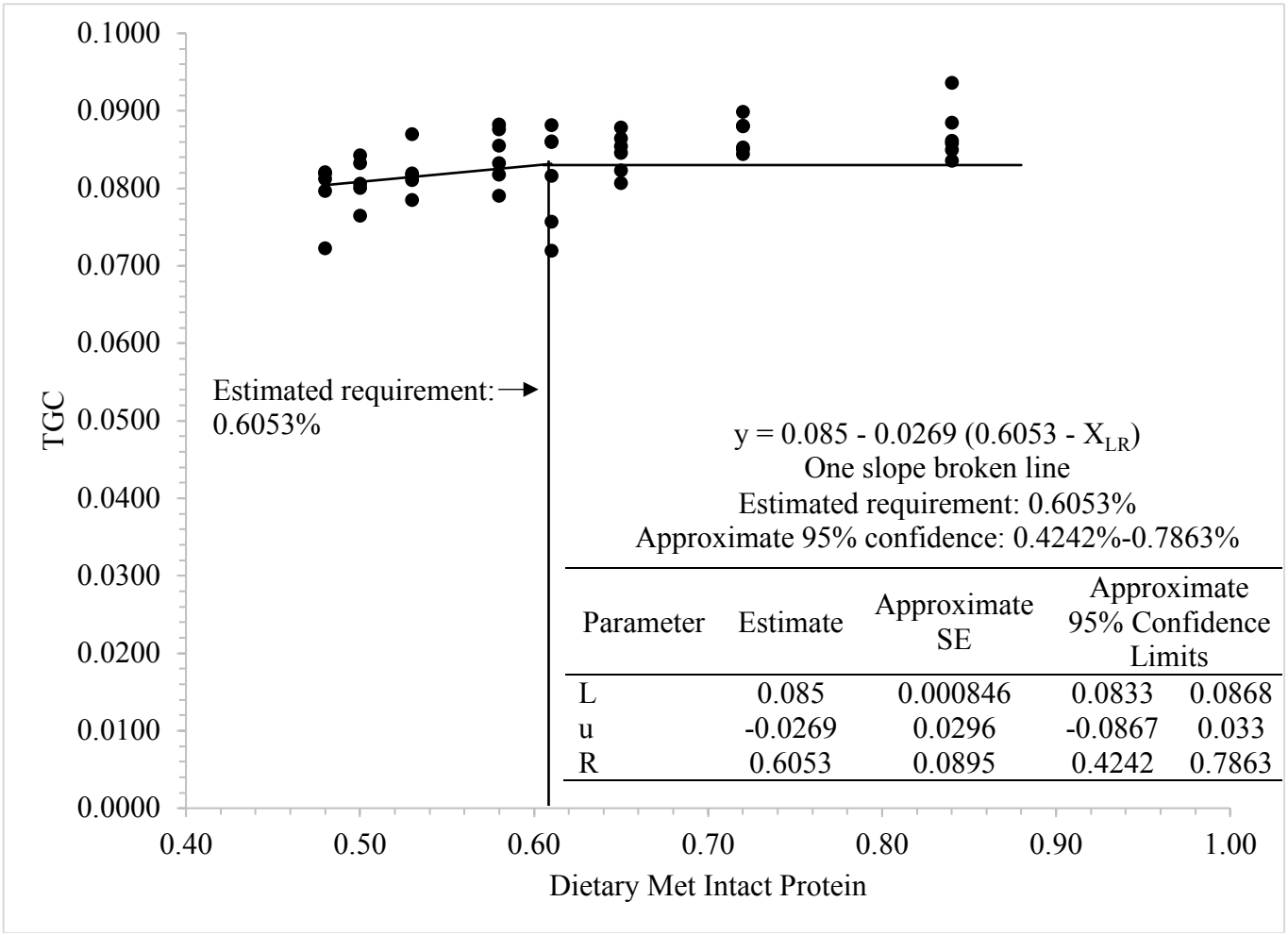


Figure 4: Thermal growth coefficient of juvenile Pacific white shrimp, *Litopenaeus vannamei* fed intact protein for Trial 2. The one slope line model estimated that optimal TGC was obtained with a dietary methionine requirement of 0.61% or 1.67% of protein, based on dry weight.



Parameter	Estimate	Approximate SE	Approximate 95% Confidence Limits	
L	0.085	0.000846	0.0833	0.0868
u	-0.0269	0.0296	-0.0867	0.033
R	0.6053	0.0895	0.4242	0.7863

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CHAPTER III

UTILIZATION OF DIFFERENT PROTEIN SOURCES AND INTESTINAL HISTOLOGY IN YELLOWTAIL SNAPPER, *Ocyurus chrysurus* (Bloch, 1971)

Abstract

Yellowtail snapper, *Ocyurus chrysurus* is a marine snapper that is relatively easy to spawn and rear larvae, making it a candidate for mariculture or a research model for other snapper species. Hence, there is interest in identifying diets that are appropriate for its culture. Yet the utilization of practical diets and their effects on snapper growth and body composition is poorly understood making the selection of feeds problematic. Hence, the objective of this work was simply to initiate the evaluation of alternative protein sources and determine if solvent extracted soybean meal produces enteritis. Towards this goal a 14-week growth trial (mean initial weight of 2.03 ± 0.06g) was conducted to observe the effects of protein sources, such as fishmeal (F), poultry meal (P), and solvent extracted soybean meal (SBM) on fish performance. Four diets were formulated to contain 40% protein and 10% lipid. The basal diet contained 30% fishmeal (F30) which was systematically replaced with poultry meal (F15:P15 and F0:P30) as well as a diet with reduced fishmeal and high level of soybean meal (F15:SMB40). At the conclusion of the growth trial there we no difference in growth; however, there were difference in feed utilization with the F30:P0 and F15:SBM40 diets producing the best utilization. Histological measurements of the distal intestine mucosal length, thickness of the mucosa, lamina propria, submucosa, and serosa, as well as histological scoring of the lamina propria folds, connective tissue, and large vacuoles showed no significant differences ($p > 0.05$) among fish fed F30:P0, F0:P30, and F15:SMB40. Results showed

no adverse effects on growth performance and intestinal histology in yellowtail snapper when fed diets containing reduced levels of fishmeal and 40% SBM meal.

Key words: yellowtail snapper, protein sources, fishmeal, poultry meal, soybean meal, gut histology, enteritis

1. Introduction

Increasing demand for seafood and the consequent declines in catchable wild fisheries has led to the growth of the aquaculture industry worldwide (FAO, 2022a). Marine aquatic fish and invertebrate aquaculture, or "mariculture", is now substantial sources of animal protein and have been among the fastest growing food sectors in the world over the past 20 years, with an estimated first-sale value of US$106 billion (Edwards et al., 2019; FAO, 2020; Naylor et al., 2021). Mariculture contributes 36% to the total value and 34% to the total aquaculture production, and while marine finfish comprises only a portion of global aquaculture production, its share is increasing. Mariculture holds significant promise for providing sustainable and nutritious food sources to help meet growing protein demand. There are emerging opportunities to increase mariculture production through offshore aquaculture (Costello et al., 2020). The increasing recognition of the potentially lower environmental impacts of mariculture compared with land-based animal products (Hall, 2011; Hilborn et al., 2018) provides a great opportunity for expansion. Feed has been considered as one of the major factors affecting production costs in fed based aquaculture operations. The current need to develop high quality feeds which possess complete nutritional composition to promote acceptable growth has been a constant challenge in the aquaculture industry.

Marine finfish culture has a generated the interest of aquaculturists worldwide due to its recreational and commercial fisheries potential. The potential for supplying sustainable seafood and closing the gap between supply and demand for marine fish species for human consumption. Recent studies evaluating the potential of the yellowtail snapper *Ocyurus chrysurus* (Bloch, 1791), and its culture indicate a promising reception and positive potential. Yellow tail snapper are known

to have a high market value and supports an economically important commercial and recreational fishing industries (Alvarez-Lajonchère and Ibarra-Castro, 2013).

Yellowtail snapper is widely distributed in the tropical and subtropical western Atlantic Ocean from the United States of America to Brazil (De La Morinière et al., 2003) and is one of the most important and heavily targeted reef fishery species in the Caribbean (Watson et al., 2002). This marine fish is an important fisheries resource and is considered a good saltwater species candidate for aquaculture due to advantages associated with established captive spawning methods, handling tolerance, artificial feed acceptance, stock enhancement potential, and good consumer reception in both whole-fish and fillet market applications (Soletchnik et al., 1989; Watanabe et al., 1998; Turano et al., 2000; Gutiérrez-Sigeros et al., 2018). Grow-out trials with wild-caught juvenile yellowtail snapper have indicated good adaptation to breeding conditions, rapid weaning to commercial feeds, and high survival rates (René and Haffner, 1982; Soletchnik et al., 1988). Moreover, due to its high price, demand, food quality, good consumer acceptance, crowding, and environmental tolerance, and its adaptability to practical formulated dry diets (Alvarez-Lajonchere et al., 1992; Gutiérrez-Sigeros et al., 2018) the yellowtail snapper is considered a promising candidate for aquaculture. However, information on grow-out nutrition, acceptance of different ingredients and culture studies of the yellowtail snapper are limited. Although there is considerable interest in developing culture techniques for snapper, recent attempts have been met with limited success (Davis et al., 2005).

Currently studies on yellowtail snapper are insufficient, leading to poor understanding of its culture and feed requirements. In order to initiate the development of practical diet formulations for any marine fish species, one of the first questions to answer is simply how much fishmeal is needed in an initial formulation and can other animal or plant-based proteins, such as poultry meal

and soybean meal (SBM) replace fishmeal as a protein source. The promising potential of soybean meal as a fishmeal alternative is evident due to the security of its supply, price, and reasonable amino acid profile that approaches the nutritional requirements of many cultured fish (Refstie et al., 2000; Storebakken et al., 2000). However, high soybean meal inclusion in some fish diets has been proven to trigger an inflammatory response in the distal intestine mucosa and cause negative effects on gut health (Krogdahl et al., 2010; Merrifield et al., 2011). In some species, high levels of dietary SBM has been proven to induce intestinal enteritis, increase inflammatory immune responses, and influence the intestinal microbe composition (Rumsey et al., 1994; Heikkinen et al., 2006; Venold et al., 2012). This condition, called soybean meal-induced enteritis (SBMIE) disrupts gut integrity, slows growth, and eventually leads to mortality in susceptible fish species. SBMIE has been reported in common carp (Urán et al., 2008c), zebra fish (Hedrera et al., 2013), rainbow trout causing negative impacts on performance, gut health, and membrane integrity (Komatsu et al., 2009), and in Atlantic salmon inducing distal intestine enteritis (Van den Ingh et al., 1991; Urán et al., 2009; Munang'andu et al., 2012).

The current study aimed to evaluate the response of yellowtail snapper to practical diets containing different protein sources including fishmeal, poultry meal, and soybean meal. In addition, this study aimed to determine the possibility of soybean meal induced enteritis (SBMIE) in yellowtail snapper when fed with high soybean meal diets, using gut histological measurements and scoring.

2. Materials and Methods

2.1 Experimental Design and Diets

Diets were formulated to be isonitrogenous and isolipidic (40:10) and in accordance with the nutritional requirements of marine fish (NRC, 2011). In the first three diets, fishmeal was incrementally replaced with poultry meal. The diets consisted of 30% fishmeal and 0% poultry meal (F30:P0), 15% fishmeal and 15% poultry meal (F15:P15), and 0% fishmeal and 30% poultry meal (F0:P30). The fourth diet was formulated to contain 15% fishmeal and 40% soybean meal (F15:SBM40) to evaluate the possibility of enteritis issues. The diet formulation and proximate composition of the experimental diets are presented in Table 1. The Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL) prepared the experimental diets using standard procedures for fish feeds. The dry ingredients and oil were weighed and mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Then, boiling water was then blended into the mixture to obtain a consistency appropriate for pelleting. The moist pellets were pressure-pelleted using a meat grinder with a 3-mm die. The pellets were then placed into a forced air oven (<45°C) overnight and were crumbled, packed, and stored in sealed bags in a freezer (-20°C) after drying. Proximate composition analysis of all experimental diets was performed at MidWest Laboratories, Inc., Omaha, NE, USA (Table 1).

2.2 Growth Trial

The growth trial was conducted at the E.W. Shell Fisheries Center at Auburn University, Auburn, Alabama, in an indoor recirculation system consisting of twenty-four 730-L polyethylene circular tanks connected to a common reservoir tank (1600-L), fluidized biological filter, bead filter and circulation pump. Thirty (30) juvenile yellowtail snapper were batch-sorted, group

weighed, and haphazardly stocked into each culture tank at a stocking density of 1 fish per 24 L of water. Yellowtail snapper for the experiments were obtained from the University of Miami Experimental Hatchery (UMEH) via the Whitney Laboratory for Marine Bioscience at University of Florida.

During the growth trial, three replicate groups per dietary treatment were randomly assigned to dietary treatments and the feed was offered to the fish divided into two equal feedings daily. To obtain biometric data and adjust the daily feed ration, every other week all of the fish in each tank were weighed together and then counted to determine the average weight. This data and observations of feeding response were then used to adjust feed inputs which were calculated based on a fixed percent of weight. During each sampling, fish were dipped in a solution of chloroquine phosphate (MP Biomedicals, Solon, OH) at a concentration of 60 mg/L for about 2 min followed by a 10-15 s dip in unchlorinated freshwater, to minimize possible amyloodinium (*Amyloodinium ocellatum*) infections. At the end of the 14-week growth trial, fish were counted, and group weighed by replicate tank to determine the mean final biomass, final weight, survival, weight gain, thermal-unit growth coefficient (TGC), and feed conversion ratio (FCR). A haphazard sample of 6 fish were euthanized using 100 ppm buffered tricane methanesulfonate (MS-222) and then used for morphological indices and histology (3 fish). The remaining 3 fish were packed in sealed bags and stored in a freezer at -20°C for proximate analysis.

2.3 Histology

As previously mentioned, three fish per tank were euthanized and dissected to obtain a 2 cm long section of the distal intestine to assess any soy-induced changes based on the protocols of Buttle et al. (2001); Sealey et al. (2013). Distal intestine samples were then fixed in 10% buffered

formalin for 24 hours and transferred to ethyl alcohol for long-term storage for later histological analysis. The samples were then trimmed and placed in cassettes with 70% ethanol and sent to the Auburn University Veterinary School for processing. After embedding and processing, cross-sections of the distal intestine were stained with hematoxylin and eosin (H&E) using standard histological techniques (Bureau et al., 1998; Burrells et al., 1999).

All slides and micrographs were examined using a transverse light microscope (Nikon E-200, Melville, NY, USA) at 100x magnification, and the intestinal morphology segments were evaluated using previously reported methodology and an ordinal scoring criteria system based on lamina propria thickness and cellularity, submucosal connective tissue width, and the number of large vacuoles [Barnes et al. (2014)]; Table 5). Two separate reviewers independently analyzed all slides at random and assigned a ranking to each slide, based on the overall intestinal appearance and composition. Slides with a ranked difference of ±4 were re-examined by both reviewers for confirmation. Assessed ranks were compiled and averaged for overall gut scoring. For gut histological measurements, the diameter (D), mucosal length (ML), mucosal thickness (MT), lamina propria thickness (LP), mucosal thickness/lamina propria thickness (MT/LP), submucosa thickness (SMT), and serosa thickness (ST) were measured using ImageJ v150 (NIH, Bethesda, MD, USA) software,

2.4 Water Analysis

Dissolved oxygen levels were kept near saturation using air stones in each culture tank and the sump tank, which were connected to a regenerative blower via common airline. Throughout the trial, dissolved oxygen, salinity, and water temperature were measured twice daily using a YSI-55 multiparameter instrument (YSI Corporation, Yellow Springs, Ohio, USA), while total ammonia N (TAN) and nitrite-N were measured twice per week using YSI 9300 photometer (YSI,

Yellow Springs, OH). The pH of the water was measured twice weekly during the experimental period using a pHTestr30 (Oakton Instrument, Vernon Hills, IL, USA).

During the growth trial, DO, temperature, salinity, pH, total ammonia nitrogen (TAN), and nitrite were maintained within acceptable ranges for marine fish culture at 6.51 ± 0.06 mg/L, 26.34 ± 0.1 °C, 28.32 ± 0.16 g/L, 7.83 ± 0.03, 0.45 ± 0.06 mg/L, and 0.97 ± 0.19 mg/L respectively.

2.5 Statistical Analysis

All data were analyzed using SAS software (V9.4, SAS Institute, Cary, NC, USA). The parametric data as fish growth indices were analyzed using one-way ANOVA to determine significant differences ($P < 0.05$) among treatments followed by Tukey's multiple comparison test to evaluate significant differences between treatment means. To explore possible differences in the histological intestine scoring data of fish fed different protein sources, the Shapiro-Wilk normality test followed by a Kruskal-Wallis test for testing the homogeneity of variances.

3. Results

3.1 Growth performance parameters and fish body composition

No statistically significant differences between the treatments were detected in growth parameters. The numerical highest percent weight gain value was observed in fish fed F30, while the numerical lowest value was observed in fish fed P30 (Table 3). The same trend albeit significant, was observed for apparent net protein retention (ANPR%) where fish fed F30 performed best among all experimental diets. Likewise, significant differences were observed in the FCR, with the highest values observed in fish fed P30, while fish fed F30 had the lowest FCR among all experimental diets. The opposite trend was observed for survival where significantly low mortalities were observed in fish fed P30 while fish fed F15:SBM40 had the poorest survival.

No significant differences were observed for all proximate composition of fish fed experimental diets containing different protein sources (Table 4).

3.2 Intestinal histology evaluation

Histological measurements of distal intestine (Table 5) showed no significant difference in diameter (D), mucosal length (ML), mucosal thickness (MT), lamina propria thickness (LP), mucosal thickness/lamina propria thickness (MT/LP), submucosa thickness (SMT), and serosa thickness (ST), as well as in the histological scoring (Table 6) of the lamina propria folds, connective tissue, and large vacuoles among fish fed F30, P30, and F15: SMB40.

4. Discussion

4.1 Growth performance parameters and fish body composition

There is considerable interest in culturing yellowtail snapper, but there is limited information regarding their response to primary protein sources which complicates the selection or design of appropriate feeds. Hence, for this work we simply look at the acceptability of two common protein sources poultry by product meal and soybean meal. In this research, there was no significant differences in growth; however, there was a clear reduction in growth as fishmeal was removed. Fishmeal is viewed as an ideal protein source for (Olsen and Hasan, 2012). Yellowtail snapper fed diets containing 30% poultry meal (P30) performed poorest among all tested protein sources for all growth performance parameters, except for survival. As would be expected based on growth, the poorest FCR and ANPR values were observed in yellowtail snapper fed a high poultry meal diet, which were significantly different in fish offered the fishmeal-based diet. This is in contrast to other studies suggesting poultry meal can completely replace fishmeal in marine fish such as red snapper (*Lutjanus campechanus*) (Walsh et al., 2021), spotted red nose snapper

(*Lutjanus purpureus*) (Hernández et al., 2014), and gilthead sea bream (*Sparus aurata*) (Alexis et al., 1999). A meta-analysis also revealed no significant difference detected between poultry meal supplemented diets and 100% fishmeal control diets for most species (Galkanda-Arachchige et al., 2020). The authors noted that varying responses are likely attributed to differences in the quality, source, and digestibility of the product utilized. This is also likely the case in this example, with poultry meal possibly having lower digestibility resulting in a slight reduction of growth and notable difference in feed utilization.

Due to its worldwide availability, security of supply, price, and reasonable amino acid profile soybean meal is a common protein source in commercial feed formulations (Refstie et al., 2000; Storebakken et al., 2000). A slight, albeit not statistical, reduction in weight gain (%) for yellowtail snapper fed the high soybean meal diet was observed when compared to fish fed diets containing fishmeal, specifically F30 and F15:P15, which would indicate a slight issue with nutrition or palatability of the feeds. Significant reduction in fish performance was observed in the spotted red nose snapper (*Lutjanus guttatus*) when fed 60% SBM replacement of fishmeal (Silva-Carrillo et al., 2012), as well as in red snapper (*Lutjanus campechanus*) when fed high soybean meal diets, primarily caused by palatability reduction issues (Davis et al., 2005). In other cases, e.g. trout and other species that do not tolerate high levels of SBM the response often due to anti-nutritional factors and/or an allergic response. However, when using soy sources that are processed to remove antinutrients a poor response may still be observed. For example, Barnes et al. (2014) reported that rainbow trout demonstrated a decline in rearing performance when fed diets containing 40% or more fermented soybean meal PepSoyGen. Additionally, Yamamoto et al. (2010) described poor rainbow trout performance when using diets having 47.6% fermented soybean meal supplemented with seven amino acids. However, results have also been observed in

rainbow trout where no negative growth effects were observed, despite soy-induced intestinal damage during 56- and 84-d feeding trials (Bureau et al., 1998). Hence, the presence of enteritis is not always accompanied by a growth response under short term growth trials as exhibited by the aforementioned study.

Fishmeal has been the protein source of choice in diets for farmed fish since the protein content of fishmeal is relatively high, generally reaching 65 to 72 %, depending upon the fish species used to produce it. Another reason is its high palatability, low anti-nutritional factors, and high protein and amino acid apparent digestibility and (Sugiura et al., 1998; Cerqueira et al., 2020; Lim et al., 2023). A study on determination of protein quality (evaluating single protein feedstuffs in otherwise nutritionally complete diets) proved that at lower dietary crude protein concentrations, significant differences existed between soybean meal and fishmeal, suggesting inferior protein quality when using only soybean meal as the protein source (Davis and Stickney, 1978).

It is clear that when replacing fishmeal, which is not only an excellent nutrient source but also a palatability enhancer, that several issues can occur depending on the replacement strategy and diet matrix. Reduced performance could be attributed to a minor deficiency of EAA, as poultry meal and soybean meal are low in several EAA such as taurine and methionine which are an essential part of the diet for many marine teleosts (Yokoyama et al., 2001; Salze and Davis, 2015). Irrespective of the reason, the differences in performance were relatively minor indicating a reasonable tolerance to these alternative protein sources.

4.2 Intestinal histology evaluation

As was previously noted, in relatively short-term studies growth may be reasonable but enteritis may occur which will eventually lead to gut health issues. This study was conducted over a 14-week growth period in which fish exceeded weight gains of 658%, which should be adequate

to induce intestinal modifications (Table 3) and enteritis if it were to occur. Results of the present study showed no significant differences for all histological measurements (Table 5, Figure 1-2) and histological scoring (Table 6). Findings from the histological evaluation indicated no apparent enteritis in high soybean meal fed yellowtail snapper. This agrees with a study on rainbow trout where no significant differences were observed in the intestinal morphology when fed with 35-50% fermented soybean meal inclusion (Barnes et al., 2014).

Typical signs of SBMIE, firstly described by Van den Ingh et al. (1991), include reduction in mucosal fold height; disappearance of supranuclear vacuolization; thickening of both lamina propria and sub-epithelia mucosa with a severe infiltration of inflammatory cells (Baeverfjord and Krogdahl, 1996; Van den Ingh et al., 1996; Krogdahl et al., 2003; Urán et al., 2008b). Intestinal changes are likely to occur very rapidly and then either continue to deteriorate or stabilize depending on the amount of soybean meal in the diet (Urán et al., 2009). In several studies, SBMIE has been qualitatively assessed by describing histological alterations of the distal intestine, which is one of the methods conducted in the current study, in addition to intestinal histology morphometric measurements. In Atlantic salmon, the fully developed condition was characterized by shortened heights of the mucosal foldings; loss of the normal supranuclear vacuolization of the absorptive cells in the intestinal epithelium; widened central stroma within the mucosal foldings, with increased amounts of connective tissue; and profound infiltration of inflammatory cells in the lamina propria (Baeverfjord and Krogdahl, 1996). The observed negative effects of soybean meal include proliferative or inflammatory conditions in the distal intestinal mucosa of cultured fish species such as rainbow trout (Rumsey et al., 1994), common carp (Urán et al., 2008a), juvenile totoaba, *Totoaba macdonaldi* (Fuentes-Quesada et al., 2018) and zebra fish (Hedrera et al., 2013).

For the present study, no significant inflammatory response of the intestinal mucosa was observed when fed diets with 20% to 40% inclusion of soybean meal, which is in agreement with other marine species such as Atlantic halibut *Hippoglossus hippoglossus* (Grisdale-Helland et al., 2002), European sea bass *Dicentrachus labrax* (Bonaldo et al., 2008), and turbot *Psetta maxima* (Bonaldo et al., 2011). Since no significant intestinal morphological changes were observed based on intestinal histology measurement and scoring, the current study indicates that there is no occurrence of SBMIE in yellowtail snapper fed high soybean meal diets.

5. Conclusion

In conclusion, results from this study indicate that among the protein sources examined in this study, fishmeal was a slightly better protein source for yellowtail snapper practical diets as opposed to diets containing poultry by-product meal and solvent extracted soybean meal. Differences in feed utilization may indicate a minor nutritional deficiency or a lower digestibility of these protein source. No significant intestinal morphological changes were observed in the intestinal histology morphometric measurements and scoring for yellowtail snapper fed 40% soybean meal inclusion, demonstrating that high soybean meal inclusion diets do not induce SBMIE in yellowtail snapper. As SBM did not induce enteritis and there were no clear signs of palatability issues with the diets containing lower levels of fishmeal. Overall, the response to these protein sources would indicate that the yellowtail is fairly tolerant to their inclusion in feed formulations.

Table 1: Formulation and proximate analysis of practical yellowtail snapper diets containing various protein sources of fishmeal (F), poultry meal (P), and soybean meal (SBM) (% as is). Diets were designed to contain 40% protein and 10% lipid.

Ingredient (% as is)	F30	F15:P15	P30	F15:SBM40
Menhaden fishmeal [1]	30.00	15.00	0.00	15.00
Poultry meal [2]	0.00	14.50	29.00	0.00
Soybean meal [3]	19.60	19.60	19.60	40.00
Corn protein concentrate - Empyreal 75 [4]	10.00	10.00	10.00	10.00
Menhaden fish oil [5]	5.50	5.40	4.80	3.04
Lecithin (soy) [6]	1.00	1.00	1.00	1.00
Corn Starch [7]	5.85	5.45	5.55	0.41
Wheat Flour [8]	26.00	26.00	26.00	26.00
Trace mineral premix [9]	0.25	0.25	0.25	0.25
Vitamin premix [10]	0.50	0.50	0.50	0.50
Choline chloride [7]	0.20	0.20	0.20	0.20
Rovimix Stay-C [11]	0.10	0.10	0.10	0.10
CaP-dibasic [7]	0.00	1.00	2.00	2.50
Taurine [12]	1.00	1.00	1.00	1.00
Proximate composition (g/100 g as is)[13]				
Protein (crude) %	41.6	41.7	41.6	41.6
Moisture %	8.51	7.89	7.98	8.05
Fat %	8.98	9.52	9.92	6.17
Ash %	91.49	92.11	92.02	91.95
Sulfur %	0.7	0.76	0.69	0.75
Phosphorus %	1.24	1.22	0.98	1.32
Potassium %	0.78	0.85	0.74	1.15
Magnesium %	0.15	0.15	0.12	0.19
Calcium %	1.69	1.53	0.98	1.54

Sodium %	0.28	0.24	0.14	0.18
Iron %	213	273	207	298
Manganese %	46.3	61.4	72.4	75
Copper %	4.9	8.2	9.7	8.6
Zinc %	94.9	172	218	235

[1] Special Select™, Omega Protein Inc., Houston, Texas, USA.
[2] River Valley Ingredients., 1170 Country Road 508. PO. Box 429 Hanceville, AL.
[3] Solvent Extracted Soybean Meal, De-hulled solvent-extracted soybean meal, Bunge Limited, Decatur, AL, USA.
[4] Empyreal 75 TM Cargill Corn Milling, Cargill Inc., Blair, Nebraska, USA.
[5] Omega Protein Inc., Reedville, Virginia, USA.
[6] The Solae Company, St. Louis, MO, USA.
[7] MP Biomedicals Inc., Solon, OH, USA.
[8] Bobs Red Mill Natural Foods, Milwaukie, OR, USA.
[9] Trace mineral premix (g/100g premix): cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulphate 4.0, magnesium sulphate anhydrous 13.862, monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulphate heptahydrate 13.193, filler 67.964.
[10] Vitamin premix (g/kg premix): Thiamin HCl0.751, riboflavin4.505, pyridoxineHCl1.502, D-Pantothenic acid hemicalcium salt7.508, nicotinic acid 7.508, biotin 0.075, folic acid 0.270, vitamin B12 0.003, inositol 7.508, menadione 3.003, vitamin A acetate (500,000 IU/g) 0.300, vitamin D3 (1,000,000 U/g) 0.60, DL- α -tocopheryl acetate (250/ IU g-) 12.012, α -cellulose 804.847.
[11] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, New Jersey, USA.
[12] TCI (Tokyo Chemical Industry), Portland, OR, USA.
[13] Analysis was performed by Midwest Laboratories, Inc., Omaha, NE, USA.

Table 2: Histological scoring system used on yellowtail snapper fed diets containing various protein sources during a 14-week culture period (Barnes et al. 2014).

Score	Appearance
	Lamina propria of simple folds
1	Thin and delicate core of connective tissue in all simple folds.
2	Lamina propria slightly more distinct and robust in some of the folds.
3	Clear increase in lamina propria in most of the simple folds.
4	Thick lamina propria in many folds.
5	Very thick lamina propria in many folds.
	Connective tissue between base of folds and stratum compactum
1	Very thin layer of connective tissue between base of folds and stratum compactum.
2	Slightly increased amount of connective tissue beneath some of the mucosal folds.
3	Clear increase of connective tissue beneath most of the mucosal folds.
4	Thick layer of connective tissue beneath many folds.
5	Extremely thick layer of connective tissue beneath some of the folds.
	Vacuoles
1	Large vacuoles absent.
2	Very few large vacuoles present.
3	Increased number of large vacuoles.
4	Large vacuoles are numerous.
5	Large vacuoles are abundant and present in most epithelial cells.

Table 3: Response of juvenile yellowtail snapper (mean initial weight = 2.03 ± 0.06 g) fed diets containing various protein sources during a 14-week period. Values represent the means of three replicates.

	Final Biomass (g)	Final weight (g)	Weight Gain (g)	Weight Gain (%)	Total dry feed per fish (g)	FCR[1]	Survival (%)	TGC[2]	ANPR[3] (%)
F30	390.8	15.40	13.37	658	32.51	2.43 [b]	84 [a,b]	0.0473	18.89 [a]
F15:P15	347.3	13.95	11.99	611	30.59	2.58 [a,b]	82 [a,b]	0.0445	17.36 [ab]
P30	325.8	11.95	9.95	499	28.97	2.92 [a]	91 [a]	0.0397	16.02 [b]
F15:SBM40	291.7	13.04	10.97	529	28.02	2.56 [a,b]	74 [b]	0.0418	18.47 [a]
PSE[4]	17.56	0.55	0.54	26.53	0.90	0.07	2.37	0.0012	0.3882
p-value[5]	0.2567	0.1415	0.1276	0.1035	0.3375	0.033	0.0471	0.1199	0.009

[1]FCR = Feed conversion ratio = feed offered / (final weight-initial weight).
[2]TGC = Thermal-unit growth coefficient.
[3]ANPR = Apparent net protein retention.
[4]PSE = Pooled standard error.
[5]One-way analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$). Tukey's multiple comparison test was used to determine statistically significant differences between treatment means when there was statistical significance in the ANOVA test (n = 3), represented by values with different letters.

Table 4: Whole-body composition (on wet weight basis) of yellowtail snapper fed diets containing varying protein sources during a 14-week culture period.

	Moisture %	Protein (crude) %	Fat %	Ash %
F30	68.17	18.67	6.69	4.85
F15:P15	68.47	18.27	7.68	4.58
P30	68.30	18.90	6.75	4.85
F15:SBM40	69.67	19.07	5.56	4.94
PSE[1]	0.43	0.25	0.37	0.17
p-value[2]	0.6439	0.7563	0.2746	0.9233

[1]PSE = Pooled standard error.
[2]One-way analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$). Tukey's multiple comparison test was used to determine statistically significant differences between treatment means when there was statistical significance in the ANOVA test (n = 3), represented by values with different letters.
Analysis was performed by MidWest Laboratories, Inc., Omaha, NE, USA.

Table 5: Histological measurements (μm) of the distal intestine of yellowtail snapper fed diets containing various protein sources. Values represent means of three replicates. One-way analysis of variance and Tukey post-hoc test was used to determine significant differences ($P < 0.05$) among treatments (n = 3), represented by values with different letters.

	Diameter	Mucosal Length	Mucosal Thickness	Lamina Propria Thickness	MT/LP	Submucosa Thickness	Serosa Thickness
F30	3262.17	1347.16	384.13	158.93	2.73	175.73	120.34
P30	2781.94	1267.78	350.19	145.99	2.64	197.75	120.53
F15:SBM40	2645.52	1343.38	369.78	193.70	1.91	195.76	118.37
PSE	120.13	118.64	23.23	12.32	0.16	17.42	9.58
p-value	0.0855	0.9562	0.8437	0.2691	0.0685	0.857	0.9951

Table 6: Mean (±SE) of distal intestine morphological scores from yellowtail snapper fed diets containing various protein sources.

	Lamina propria of simple folds		Connective tissue at base of folds		Large vacuoles	
F30	1.85	±0.13	1.21	±0.09	1.75	±0.21
P30	2.19	±0.23	1.42	±0.09	2.13	±0.23
F15:SBM40	2.63	±0.24	1.31	±0.10	2.04	±0.17
H (2)	5.0079		3.2404		2.4731	
p-value[1]	0.0818		0.1979		0.2904	

[1]Note: Kruskal-Wallis test was used to determine statistically significant differences ($p < 0.05$) between treatment means (n = 3).

Figure 1: Example cross section of the distal intestine of yellowtail snapper fed diets containing varying protein sources. The diameter (4×) of intestines was measured from one point at the edge of serosa to another point at the edge of the serosa to another point at the edge of the serosa horizontally. Scale bar 100 μm, H.E., ×4.

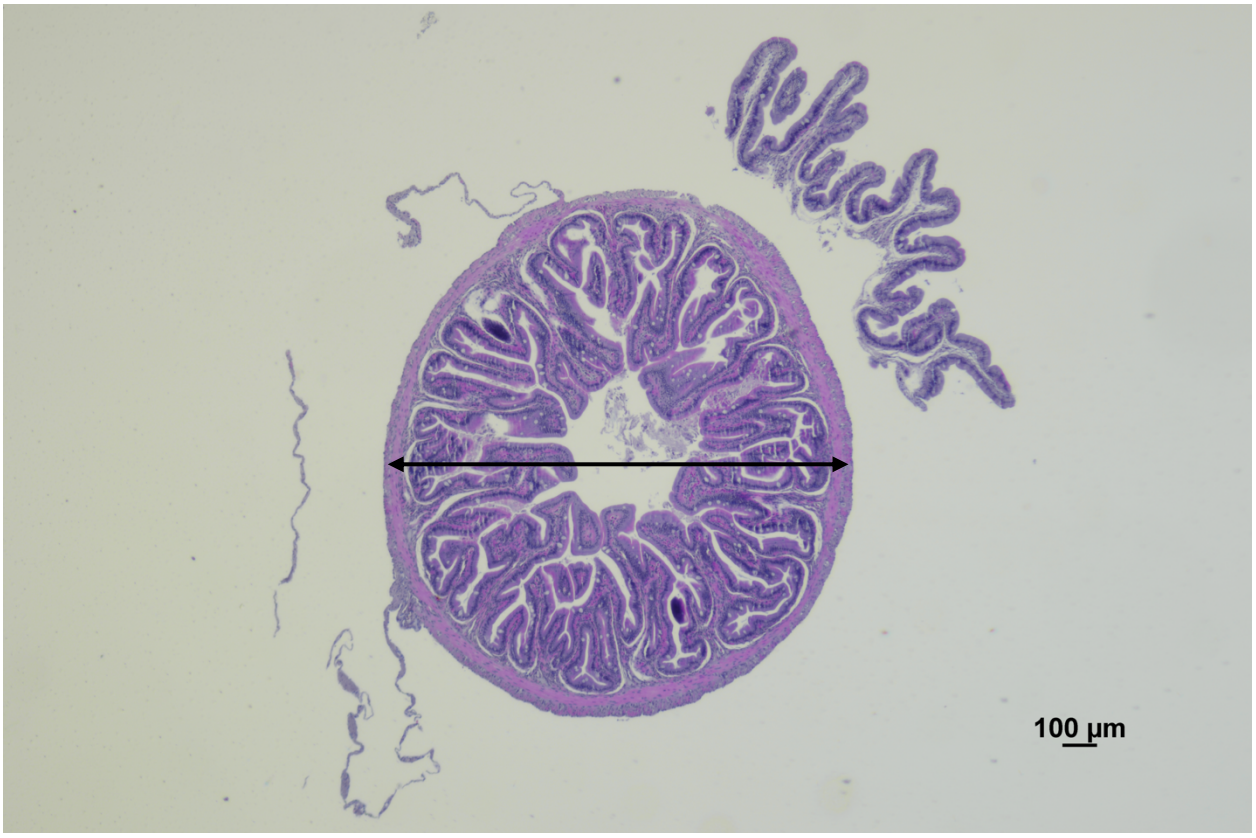
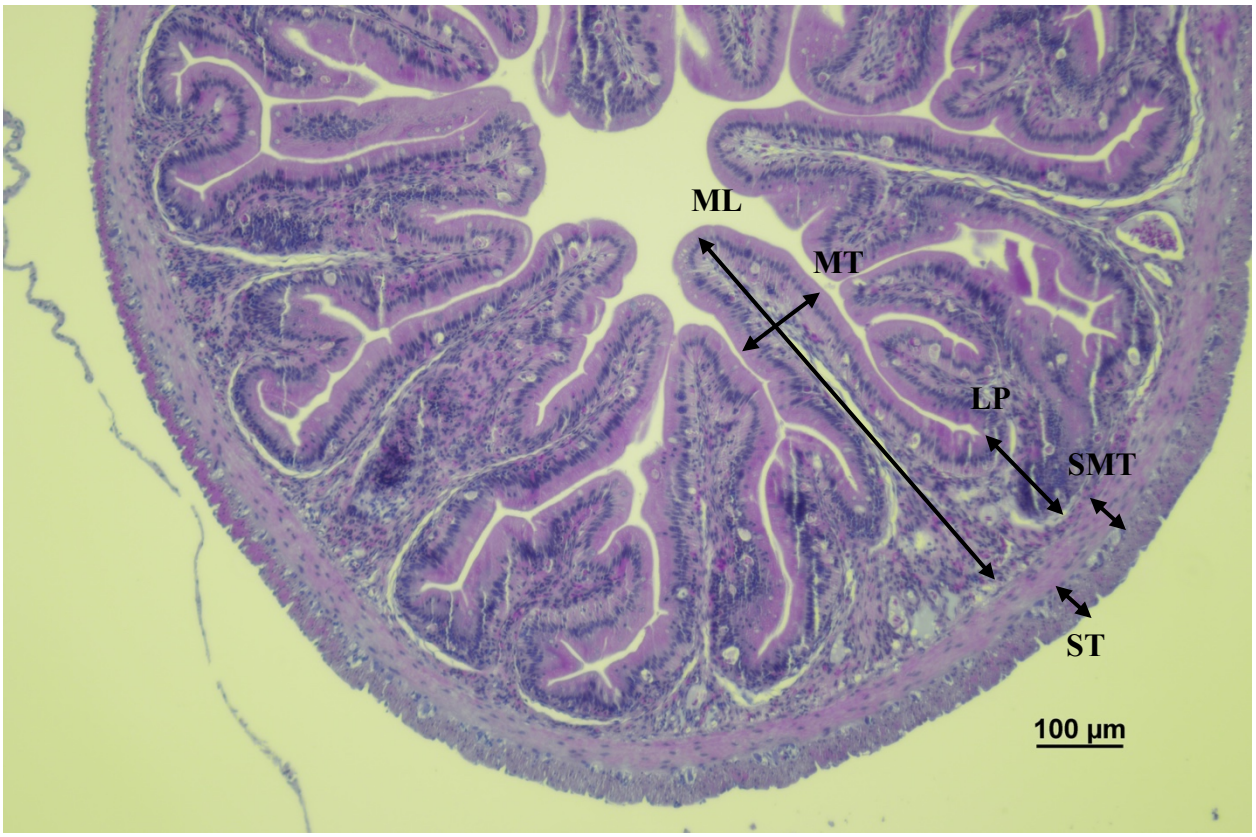


Figure 2: Example cross section of the distal intestine of yellowtail snapper fed diets containing varying protein sources. The length of the mucosa (10×) was measured from the tip of the mucosal fold to the inner tip of the stratum compactum (ML). Mucosal thickness (MT), lamina propria thickness (LP), submucosa thickness (SMT), and serosa thickness (ST) were measured as labelled in the figure. Scale bar 100 μm, H.E., ×10.



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CHAPTER IV

EFFECTS OF DIFFERENT PROTEIN AND LIPID LEVELS IN PRACTICAL DIETS FOR YELLOWTAIL SNAPPER, *Ocyurus chrysurus* (Bloch, 1971)

Abstract

Yellowtail snapper *Ocyurus chrysurus,* has considerable potential in aquaculture yet there is very limited information on nutritional requirements. To initiate base line data, two trials were conducted to evaluate the effects of dietary protein and lipid levels in practical diets on growth and protein retention for the yellowtail snapper. The first trial was conducted over a 14-week period using a series of diets having varying levels of protein (36%, 40%, and 44%) and lipid levels (6%, 10%, and 14%). The second trial was conducted for 10 weeks with a series of diets that were formulated to have 36% protein and incremental levels of lipid (7%, 10%, 13%, and 16%). Additionally, a commercial reference diet containing 44% protein and 12% lipid was included as a reference for this trial. Based on growth performance and feed utilization parameters among all protein and lipid levels for Trial 1, the yellowtail snapper was able to effectively utilize practical diets formulated to contain 36% protein and 10% lipids which produced the highest ANPR% and survival. Based on our results, we recommend 36% protein and dietary lipid levels of 7%-13%, which are lower than the currently used commercial diets for marine finfish. The knowledge gathered from the current study may be helpful for nutritionists in formulating feed based on more sustainable and cheaper feedstuffs and promote sustainable yellowtail snapper aquaculture.

Key words: yellowtail snapper, protein, lipid, poultry meal, commercial diet, protein levels, lipid levels

1. Introduction

Mariculture contributes 36% of total value and 34% of the total aquaculture production. While the overall share of farmed fish in marine finfish production has stayed lower than captured fisheries, for the species that are farmed, cultured fish dominate the seafood market. Emerging opportunities to increase mariculture production (e.g., offshore aquaculture, (Costello et al., 2020)), and increasing recognition of the potentially lower environmental impacts of mariculture compared with land-based animal products (Hall, 2011; Hilborn et al., 2018), suggest that mariculture holds significant promise for providing sustainable and nutritious food sources to help meet growing protein demand.

Dietary protein is considered to be the most expensive component in fish diets (Choi et al., 2006; Fraser and Davies, 2009). Relatively high levels in marine fish diets, make marine fish feed costly. Elevated levels of protein in marine fish diets are viewed as crucial to obtain amino acids for protein synthesis and to meet energy needs (Walton et al., 1982; Jia et al., 2017; Teles et al., 2020). Aside from increasing feed costs, excessively high protein feeds can also result in poor fish growth and feed utilization (Gurure et al., 1995; Santinha et al., 1996). Excesses of dietary protein also increase nitrogenous excretion into the rearing water, resulting in pollution and higher environmental costs (Cho and Bureau, 2001; Kaushik and Seiliez, 2010). Quite often in high protein diets, lipid levels are increased to provide a source of non-protein energy thus sparing protein degradation. However, high lipids do not work for all species. For example, digestibility was reduced in cobia (*Rachycentron canadum*) when fed with increasing dietary lipid levels which resulted in excess lipid deposition in the tissues (Craig et al., 2006). As there are interactions between these nutrients studies on optimizing protein and lipid levels must be

conducted for diet formulation development. Studies focusing on determining protein and lipid levels promote sustainable cultivation for promising fish species that are good candidates for aquaculture, thereby diversifying the possible species options for food.

Growing interest has accrued in the possible culture of the yellowtail snapper, *Ocyurus chrysurus*, a Lutjanid tropical reef fish with a streamlined body and a deeply forked tail. This species is distributed in the western Atlantic from North Carolina to southeastern Brazil, but is most abundant in the Bahamas, off south Florida and in the Caribbean. Fishermen seek this fish as a sport fish and regard it as a good quality food fish. Commercially, the species is caught by hook and line, baited trap, trammel and gill nets, trawl and beach seine. In the U.S. Virgin Islands and Puerto Rico, the species is the most prized snapper (Manooch III and Drennon, 1987). Yellowtail snapper is an important fisheries resource where they occur, with high production in northeast Brazil (Costa et al., 2003) by averaging 1683 tons in 2001 (Rezende et al., 2003), and accounted for 16% of the total fish biomass captured between 1986-1989 (Paiva and Andrade-Tubino, 1998) in southeast Brazil. Biological research on yellowtail snapper has not been extensive and has immense potential to support a promising mariculture industry.

Nutritional information on snapper species is quite limited. There are a few studies on the use of alternative protein and lipid sources to evaluate digestibility and growth performance of snapper species such as Australian snapper were conducted using a large range of protein and lipid sources and levels (Quartararo et al., 1998; Glencross et al., 2003; Glencross and Hawkins, 2004; Booth et al., 2012). Research with the red snapper utilized different protein sources for fishmeal replacement, the use of attractants, and taurine removal (Walsh et al., 2021). Overall these studies demonstrated wide acceptability of snapper species to different protein sources.

Presently, there is very limited information on nutrient recommendations for snapper in general and almost none on the yellow tail snapper. In order to promote enhanced production and culture of yellowtail snapper, basic data on dietary requirements need to be developed. Hence this study was designed to evaluate nutritional responses of yellowtail snapper using practical diets designed to contain varying levels of protein and/or lipids.

2. Materials and Methods

2.1 Experimental Design and Diets

Two trials were conducted to determine basic protein and lipid levels for practical diets for yellowtail snapper (Table 1). For the first growth trial, five diets were formulated to cover the typical range of protein and lipids that are used in marine fish diets. This included three diets with 36, 40 and 44% protein with 10% lipid (36:10, 40:10 and 44:10, respectively). An additional two diets were formulated with 44% protein and 6 and 14% lipid. Thus, allowing three diets with 44% protein and 6, 10 and 14% lipids (44:6, 44:10 (from the first set), and 44:14). Based on results of the first trial, the second series of diets were formulated to contain 36% protein and 7, 10, 13, and 16% lipid. A commercial diet, Otohime EP3 (Reed Mariculture) was used as a commercial reference for the second series of diets.

All diets were formulated in accordance to the nutritional requirements of marine fish (NRC, 2011) and applied to yellowtail snapper. The experimental diets were prepared at the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL), using standard procedures for fish feeds. The dry ingredients and oil were weighed and mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Subsequently, boiling water was then blended into the mixture to obtain a consistency

appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die. The moist pellets were then placed into a forced air oven (VWR Scientific E191047, PA, USA) (<45°C) overnight and were crumbled, packed, and stored in sealed bags in a freezer (-20°C) after drying. Proximate composition analysis for all experimental diets were analyzed at MidWest Laboratories, Inc., Omaha, NE, USA (Table 1).

2.2 Growth Trial

Both growth trials were conducted at E.W. Shell Fisheries Center at Auburn University, Auburn, Alabama in an indoor recirculation system consisting of culture tanks, reservoir, fluidized biological filter, bead filter, circulation pump and supplemental aeration. For Trial 1, fifteen 730-L polyethylene circular tanks connected to a common reservoir tank (1600-L) were used, with thirty juvenile yellowtail snapper that were batch-sorted to uniform size, group weighed and stocked into each tank. For trial two, twenty 83-L glass rectangular tanks connected to a common reservoir tank (800-L) were stocked with ten juvenile yellowtail snappers per tank, following the described protocol. Yellowtail snapper for the experiments were obtained from the University of Miami Experimental Hatchery (UMEH) via the Whitney Laboratory for Marine Bioscience at University of Florida.

For each growth trial, three replicate groups of fish per dietary treatment were randomly assigned the test diets. The fish were offered a fixed ration that was divided into two equal feedings each day. Fish were counted and weighed every other week to adjust the daily feed ration, which was calculated based on percentage body weight which took into account the expected growth and the observed feed response. During the weighing process, fish were dipped in a solution of chloroquine phosphate (MP Biomedicals, Solon, OH) at a concentration of 60 mg/L for around 2 min followed by a 10-15 s dip in dechlorinated freshwater. This treatment was used to reduce the

likelihood of amyloodinium (*Amyloodinium ocellatum*) infections. At the end of the 14-week growth trial, fish were counted, and group weighed by replicate tank to determine mean final biomass, final weight, survival, percent weight gain, feed conversion ratio (FCR), and thermal-unit growth coefficient (TGC). Fish were euthanized and packed in sealed bags and stored in a freezer (-20°C) for proximate analysis.

2.3 Water Analysis

Dissolved oxygen was maintained near saturation using air stones in each culture tank and the sump tank each connected via a common air distribution system and a regenerative blower. During the trial, dissolved oxygen, salinity, and water temperature were measured twice daily using a YSI-55 multiparameter instrument (YSI corporation, Yellow Springs, Ohio, USA), and total ammonia N (TAN) and nitrite-N were measured twice per week using YSI 9300 photometer (YSI, Yellow Springs, OH). The pH of the water was measured twice weekly during the experimental period using a pHTestr30 (Oakton Instrument, Vernon Hills, IL, USA).

During the growth period for Trial 1, DO, temperature, salinity, pH, total ammonia nitrogen (TAN), and nitrite were maintained within acceptable ranges for marine fish culture at 6.51 ± 0.06 mg/L, 26.34 ± 0.1 °C, 28.32 ± 0.16 g/L, 7.83 ± 0.03, 0.45 ± 0.06 mg/L, and 0.97 ± 0.19 mg/L respectively. While for Trial 2, DO, temperature, salinity, pH, total ammonia nitrogen (TAN), and nitrite were maintained as follows: 7.24 ± 0.1 mg/L, 27.15 ± 0.11°C, 23.75 ± 0.33g/L, 7.89 ± 0.06, 0.47 ± 0.07mg/L, and 0.75 ± 0.17mg/L, respectively.

2.4 Statistical Analysis

All data were analyzed using SAS (V9.4, SAS Institute, Cary, NC, USA). Growth indices of fish were analyzed using one-way ANOVA to determine significant differences ($P < 0.05$)

among treatments followed by Tukey's multiple comparison test to evaluate significant differences between treatment means.

3. Results

For Trial 1, no significant differences were observed for all growth performance parameters (Table 2). For fish offered diets with various protein levels (36-44%) with 10% lipid, final weight (14.45 to 15.4 g), weight gain (12.34 g to 13.37 g or 583% to 658%), and TGC (0.0445 to 0.0473) were numerically lower for fish offered the 36% protein diet and higher for the 40% protein diet, respectively. Final biomass (390.77 to 396.83 g) and survival (84.4 % to 87.8%) were reversed as fish offered the 40% protein diet were numerically smaller than those offered the 44% protein diet, respectively. For fish offered diets with various lipid levels (6-14%) with 44% protein, final weight (13.2 to 14.85 g), weight gain (11.24 g to 12.84 g or 572% to 648%), TGC (0.043 to 0.0463), final biomass (317.1 g to 400.67 g) and survival (80% to 90%) were numerically lower for fish offered the 6% lipid diet and higher for the 14% lipid diet, respectively.

No significant differences were observed for all proximate composition parameters (Table 3) such as moisture, crude protein, fat, and ash for all yellowtail snapper fed experimental diets with varying protein and lipid levels.

For Trial 2, no significant differences (Table 4) were observed for all growth parameters, except ANPR. Highest weight gain was observed in yellowtail snapper fed the 13% lipid diet and the lowest weight gain was observed in those fed with the 16% lipid diet. For total dry feed offered per fish, the highest values were observed for yellowtail snapper fed commercial feed having 48% protein and 10% lipid, followed by fish fed 13% lipid diet and the fish that had the least amount of feed offered were those fed with 16% lipid diet. For TGC, the highest values were demonstrated

by fish fed the commercial feed, while among the experimental diets, yellowtail snapper fed 13% lipid diet had the highest TGC and fish fed 16% lipid diet had the lowest TGC. Yellowtail snapper fed commercial feed had the best FCR, while among the experimental diets, fish fed 7% lipid diet and 13% lipid diet had the best FCR and 16% lipid diet had the poorest FCR. Significant differences were observed in apparent net protein retention values (ANPR%) of yellowtail snapper. Yellowtail snapper fed 7% lipid diet had the highest, as opposed to those fed the 16% lipid diet and commercial feed had the lowest protein retention values. Significant differences (Table 5) were observed for moisture and fat for whole-body composition of yellowtail snapper when fed experimental diets.

4. Discussion

Understanding and exploring nutritional requirements is crucial in the formulation and optimization of diet development for any species. Given the limited data on yellowtail snapper, the current paper aimed to establish baseline data as initial information for yellowtail snapper nutrition. In the present study, and the exception of ANPR in Trial 2, no significant differences were observed in any growth parameters across all experimental diets with varying protein and lipid levels. However, ANPR was observed to decrease as protein levels increased, with the highest levels observed in the 7% lipid diet and the lowest in the 16% lipid diet. Although not significantly different, yellowtail snapper fed 40% protein had the highest weight gain % and total dry feed offered per fish (feed intake) as opposed to fish fed 44% protein, despite the fact of it having higher protein content (Trial 1). The lack of differences across dietary treatments would indicate that all diets supplied adequate levels of dietary protein. Dietary excesses of protein not only increase the cost of feeds but they also contribute to environmental pollution and can reduce growth. Poor

growth has been observed for other studies including those with cobia (*Rachycentron canadum*) (Chou et al., 2001), Arctic charr (*Salvelinus alpinus*) (Gurure et al., 1995) and gilthead seabream (*Sparus aurata*) (Santinha et al., 1996). This negative response is probably due to a compromise in their defense mechanisms against the possible harmful effects caused by an excessive absorption of protein and certain amino acids (Higuera et al., 1998). Interestingly, yellowtail snapper fed Pro36:Lip10 resulted in better growth performance in contrast to fish fed Pro44:Lip6, in spite of having the lowest protein content among experimental diets. This is in contrast to other studies which reported that lower protein diets can result in a deficiency of essential nutrients (Fraser and Davies, 2009; Kaushik and Seiliez, 2010), which can have an impact on protein synthesis and deposition (Peragon et al., 2001). Nonetheless, no significant differences were observed for all proximate composition parameters including crude protein, moisture, fat, and ash content of yellowtail snapper fed varying protein and lipid concentrations (Table 3). These results are in agreement with other studies conducted with marine species, such as Atlantic cod *Gadus morhua* (Morais et al., 2001) and cobia *Rachycentron canadum* (Craig et al., 2006) where wide range of protein and lipid diets were used without impacting proximate composition. Reference diets tested for snapper *Pagrus auratus* (Quartararo et al., 1998; Glencross et al., 2003; Glencross and Hawkins, 2004; Booth et al., 2012) making it difficult to formulate a standard reference diet for snapper to be used in nutritional studies, in which crude protein and lipids ranged from 500 to 700 g protein / kg diet, and 68–164 g lipid / kg diet, making recommendations on a reference diet difficult. In fact, the spotted rose snapper *Lutjanus guttatus* was reported to have good growth performance when fed diets having 470.4 to 529.4 g/kg and 88.8 to 100 g/kg protein and lipid (Abdo de la Parra et al., 2010), All of these values are relatively high when compared to the proposed levels of the current study possibly indicating over formulation for these species as well.

Various studies on marine fish have proven nutritional protein-sparing effects on growth performance and whole-body composition. A study on Australian snapper *Pagrus auratus* proved no difference in growth performance when fed a commercial diet containing 35% and 51% crude protein due to the protein-sparing effect (Quartararo et al., 1998; Chou et al., 2001; Meyer et al., 2004). In common snook *Centropomus undecimalis*, improved feed intake and protein efficiency ratio was observed in fish fed diets containing high digestive carbohydrates of 20% cornstarch, indicating a clear protein sparing effect of carbohydrates (Arenas et al., 2021). This was also reported in rainbow trout *Oncorhynchus mykiss* (Peragon et al., 1999) and silver perch *Bidyanus bidyanus* (Stone et al., 2003). Likewise, lower dietary protein levels of 32–36% were sufficient to support good growth in red snapper *Lutjanus campechanus*, where TGC values of snapper fed 44% protein were not significantly different than fish fed 36% (Miller et al., 2005; Walsh et al., 2021), which correlates to the results of the present study.

Studies on the utilization of various alternative ingredients for fishmeal replacement as a protein source have been conducted. For instance, canola protein concentrate showed no difference in growth performance of turbot when fed with 33% replacement (Nagel et al., 2014) and with 30% replacement in rainbow trout *Oncorhynchus mykiss* compared to commercial trout feed (Thiessen et al., 2003). In contrast, decreased feed digestibility was reported in spotted red nose snapper *Lutjanus guttatus* with 45% canola meal (49% protein level) as a fishmeal replacement (Hernández et al., 2020) as well as decreased SGR in Japanese seabass *Lateolabrax japonicus* when fed diets with 40% solvent-extracted canola meal (43% protein level) (Cheng et al., 2010). The presence of anti-nutritional factors (ANFs) in fishmeal alternative sources such as sinapic acid, phytic acid, tannins, and protease inhibitors, adversely affect feed palatability due to their bitter taste and astringency in turbot, *Psetta maxima* L. (Francis et al., 2001; von Danwitz and

Schulz, 2020). These ANFs may contribute to the reduction of feed intake, which eventually affects fish growth performance (Hilton et al., 1982). The same feed intake reduction can be observed in other marine fish like gilthead seabream *Sparus aurata* and turbot *Scophthalmus maximus* (Kissil et al., 2000; Nagel et al., 2014) when fed with diets containing high solvent-extracted canola meal.

Interestingly, a study on spotted rose snapper was successful in incorporating 20% soybean meal inclusion as a fishmeal alternative (Silva-Carrillo et al., 2012). Similarly, the utilization of low fishmeal diets containing a combination of soy protein concentrate and soybean meal was successful in some marine fish species such as Asian seabass *Lates calcarifer*, silver seabream *Sparus sarba*, hybrid striped bass *Morone chrysops* × *Morone saxatilis* and cobia *Rachycentron canadum* (El-Sayed, 1994; Gallagher, 1994; Boonyaratpalin et al., 1998; Salze et al., 2010). It is also remarkable to note that good feed acceptance to low 5% fishmeal inclusion diets was observed in red drum (McGoogan and Gatlin, 1997).

The assimilation and use of dietary protein for growth is affected by several factors such as energy and/or lipid levels. Studies focusing on lipid utilization demonstrated reduction of digestible protein to digestible energy ratio (DP:DE) in cobia *Rachycentron canadum*, when fed increasing dietary lipids, resulting in tissue lipid deposition (Craig et al., 2006). For Trial 2, yellowtail snapper fed Pro36:Lip13 performed best among all experimental diets (when excluding the commercial diet), and better than fish fed Pro36: Lip16 in spite of it having higher lipid content.

Dietary nutrients have a direct relationship with nutrient accumulation in tissues (Pham and Fotedar, 2017). Excessively high dietary lipids in cobia *Rachycentron canadum* caused a reduction in protein and increased lipid levels in whole body and liver tissues (Wang et al., 2005).

Highly vacuolized livers were observed in mangrove red snappers *Lutjanus argentimaculatus* (Catacutan and Pagador, 2004), which should be considered when discerning optimal lipid inclusion levels for formulated snapper diets. In contrast, lower fat content was observed in tin foil barb, *Barbodes altus* (when fed with 42% protein level and 12% lipid level) (Elangovan and Shim, 2000) and spotted red nose snapper *Lutjanus guttatus* (when fed extracted canola meal diets with 49% protein level and 13% lipid level) (Hernández et al., 2020). The low content of fat, high moisture, and ash content in the final carcass of fish fed high canola meal content diets were likely the result of reduced feed intake and weight gain of these fish (Hernández et al., 2020).

Poor feed utilization indices such as increased FCR and decreased PER were observed in spotted rose snapper when fed with high canola inclusions greater than 45%. This poor performance may be indicative of lower energy digestibility and dry matter, primarily caused by high fiber content accelerating bolus passage and reduction of intestinal retention time. Thus, reducing utilization of energy in the diet, which, in turn, could affect protein sparing and decrease growth rate (Higgs et al., 1983; Hernández et al., 2020). Lower total dry feed offered per fish for yellowtail snapper fed Pro44:Lip6 (Trial 1) may be attributed to high soybean meal replacement. This low feed acceptance was proven to induce palatability issues (Meilahn et al., 1996) in red drum *Sciaenops ocellatus* (Reigh and Ellis, 1992; Davis et al., 1995), discus *Symphosodon aequifasciata* (Chong et al., 2003), and Asian seabass *Lates calcarifer* (Boonyaratpalin et al., 1998; Tantikitti et al., 2005) when fed with high or complete soybean meal replacement.

Commercially produced feeds may have different physical and chemical characteristics from laboratory produced feeds, such as water stability and durability, pellet hardness and nutrient bioavailability, which may potentially influence feed intake and feed utilization (Peragon et al., 2001; Booth et al., 2012). Commercial floating pellets have been available for more than a decade

but recently, a larger range of size and formulations have been marketed (Bhujel et al., 2001). Actual price and amount of commercially produced feeds vary widely depending on the market and the region in the world where it was produced. Another factor that needs to be considered is feed selection, as its quality is directly associated with the seed output and the production cost (Bhujel et al., 2001). Experimental diets for Trial 2 were compared to a commercial marine fish feed as a reference diet due to the unavailability of a customized commercial feed formulated for yellowtail snapper. Typically, feed manufacturers have not been interested in producing specific broodfish diets because of substantially lower demand (Bromage et al., 1992). In line with this, the commercial feed, Otohime EP3 commercial feed – Pro48:Lip10 was ground following the same methods used for all other experimental diets to have similar physical characteristics.

Significant differences were observed for the total dry feed offered per fish, with the highest values for yellowtail snapper fed commercial feed (Pro48:Lip10). This may be due to the high amount of fishmeal and other marine products that was incorporated in the feed, making it very palatable and likable for the fish. Nonetheless, the same general trend was still observed, as when considering the experimental diets (excluding the commercial diet), the best growth performance is observed for yellowtail snapper fed Pro36: Lip13 and the least growth performance is demonstrated in fish fed Pro36: Lip16.

5. Conclusion

Based on growth performance and feed utilization parameters among all protein and lipid levels for Trial 1, the yellowtail snapper was able to effectively utilize practical diets with Pro36:Lip10 and Pro40:Lip10. Interestingly, yellowtail snapper fed the Pro36:Lip10 diet had the highest ANPR% and survival. For Trial 2, commercial feed having Pro48:Lip10 had the best

growth performance, feed utilization, and whole-body composition, as expected. When exclusively considering the experimental diets, yellowtail snapper fed Pro36:Lip13 had the best over-all performance. The current study aimed to establish baseline data as initial dietary information for yellowtail snapper nutrition and recommends dietary protein within the range of 36%-40% and dietary lipid levels of 7%-13%, which are lower than protein and lipid levels used currently in commercial diets for marine finfish. The knowledge gathered from the current study may be helpful for nutritionists seeking to formulate feed based on more sustainable and cheaper feedstuffs and promote sustainable yellowtail snapper aquaculture.

Table 1: Diet formulation and proximate analysis of practical yellowtail snapper diets with varying protein and lipid levels for both trials. Analysis was performed by MidWest Laboratories, Inc., Omaha, NE, USA.

		Trial 1					Trial 2			
Ingredient	Protein	36	40	44	44	44	36	36	36	36
	Lipid	10	10	10	6	14	7	10	13	16
Menhaden fishmeal [1]		26.70	30.00	35.10	35.10	35.10	26.70	26.70	26.70	26.70
Soybean meal [2]		17.44	19.60	21.56	21.56	21.56	18.00	18.00	18.00	18.00
CPC - Empyreal 75 [3]		8.90	10.00	11.00	11.00	11.00	8.60	8.60	8.60	8.60
Menhaden fish oil [4]		6.80	5.50	5.50	2.00	9.80	3.30	6.35	9.30	12.30
Lecithin (soy) [5]		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Corn Starch [6]		10.11	5.85	4.79	8.29	0.49	13.35	10.30	7.35	4.35
Wheat Flour [7]		26.00	26.00	19.00	19.00	19.00	26.00	26.00	26.00	26.00
Trace mineral premix [8]		0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin premix [9]		0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride [6]		0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Rovimix Stay-C [10]		0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
CaP-dibasic [6]		1.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00
Taurine [11]		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Proximate composition (g/100 g as is) [12]										
Protein (crude) %		38	41.6	45.1	45.3	45.1	38	38.3	37.7	37.7
Moisture %		8.84	8.51	8.53	10	9.73	5.33	4.92	4.99	4.54
Fat %		9.82	8.98	9.42	6.43	12.5	7.1	9.8	12.7	15.1
Ash %		91.16	91.49	91.47	90	90.27	9.42	9.31	9.5	9.33
Sulfur %		0.76	0.7	0.79	0.82	0.8	0.74	0.71	0.76	0.69
Phosphorus %		1.45	1.24	1.35	1.42	1.39	1.56	1.48	1.59	1.5
Potassium %		0.83	0.78	0.88	0.89	0.9	0.8	0.76	0.82	0.78

Magnesium %	0.16	0.15	0.16	0.16	0.16	0.16	0.15	0.16	0.15
Calcium %	2.06	1.69	1.91	2	2.06	2.27	2.14	2.25	2.24
Sodium %	0.29	0.28	0.34	0.34	0.35	0.29	0.28	0.3	0.28
Iron %	278	213	294	287	299	240	222	243	224
Manganese %	99.3	46.3	66.4	63.7	66.8	59.4	69.6	63.3	58.2
Copper %	7.3	4.9	6.2	6.3	6	5.6	5.2	6	5.4
Zinc %	201	94.9	170	163	164	150	132	147	112

[1] Special Select™, Omega Protein Inc., Houston, Texas, USA.
[2] Solvent Extracted Soybean Meal, De-hulled solvent-extracted soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal 75 TM Cargill Corn Milling, Cargill Inc., Blair, Nebraska, USA.
[4] Omega Protein Inc., Reedville, Virginia, USA.
[5] The Solae Company, St. Louis, MO, USA.
[6] MP Biomedicals Inc., Solon, OH, USA.
[7] Bobs Red Mill Natural Foods, Milwaukie, OR, USA.
[8] Trace mineral premix (g/100g premix): cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulphate 4.0, magnesium sulphate anhydrous 13.862, monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulphate heptahydrate 13.193, filler 67.964.
[9] Vitamin premix (g/kg premix): Thiamin HCl0.751, riboflavin4.505, pyridoxineHCl1.502, D-Pantothenic acid hemicalcium salt7.508, nicotinic acid 7.508, biotin 0.075, folic acid 0.270, vitamin B12 0.003, inositol 7.508, menadione 3.003, vitamin A acetate (500,000 IU/g) 0.300, vitamin D3 (1,000,000 U/g) 0.60, DL- α -tocopheryl acetate (250/ IU g-) 12.012, α -cellulose 804.847.
[10] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, New Jersey, USA.
[11] TCI (Tokyo Chemical Industry), Portland, OR, USA.
[12] Analysis was performed by MidWest Laboratories, Inc., Omaha, NE, USA.

Table 2: Response of juvenile yellowtail snapper (mean initial weight; 2.03 ± 0.06 g) fed diets containing various protein and lipid levels within a 14-week period for Trial 1. Values represent the means of three replicates.

Protein:Lipid	Final Biomass (g)	Final weight (g)	Weight Gain (g)	Weight Gain (%)	Total dry feed (g)	FCR[1]	Survival (%)	TGC[2]	ANPR[3] (%)
36:10	400.17	14.45	12.34	583.64	31.87	2.61	92.22	0.0445	18.94
40:10	390.77	15.40	13.37	658.47	32.51	2.43	84.44	0.0473	18.89
44:10	396.83	15.07	12.97	618.72	32.34	2.50	87.78	0.0461	16.74
PSE[4]	13.21	0.44	0.43	19.81	0.76	0.05	1.58	0.000898	0.44
p-value[5]	0.9678	0.725	0.6736	0.3464	0.9528	0.4612	0.1199	0.5045	0.0567
44:6	317.10	13.20	11.24	572.07	28.20	2.51	80.00	0.0430	18.49
44:10	396.83	15.07	12.97	618.72	32.34	2.50	87.78	0.0461	16.74
44:14	400.67	14.85	12.84	648.02	32.37	2.56	90.00	0.0463	16.44
PSE	18.77	0.49	0.49	28.38	0.89	0.06	2.34	0.00	0.57
p-value	0.1062	0.2529	0.3105	0.6103	0.0624	0.9326	0.1965	0.4799	0.313

[1]FCR = Feed conversion ratio = feed offered / (final weight-initial weight).
[2]TGC = Thermal-unit growth coefficient.
[3]ANPR = Apparent net protein retention.
[4]PSE = Pooled standard error.

[5]One-way analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$). Tukey's multiple comparison test was used to determine statistically significant differences between treatment means when there was statistical significance in the ANOVA test (n = 3), represented by values with different letters.

Table 3: Whole-body composition (on wet weight basis) of yellowtail snapper fed diets containing varying protein and lipid levels for Trial 1.

Protein:Lipid	Moisture %	Protein (crude) %	Fat %	Ash %
36:10	69.00	18.30	7.96	4.52
40:10	68.17	18.67	6.69	4.85
44:10	68.50	18.43	8.21	4.52
PSE	0.22	0.31	0.32	0.14
p-value	0.3359	0.9116	0.0896	0.6153
44:6	69.43	20.27	6.05	4.35
44:10	68.50	18.43	8.21	4.52
44:14	68.00	18.43	9.09	4.29
PSE[1]	0.31	0.59	0.58	0.23
p-value[2]	0.148	0.3983	0.006	0.9347

[1]PSE = Pooled standard error.
[2]One-way analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$). Tukey's multiple comparison test was used to determine statistically significant differences between treatment means when there was statistical significance in the ANOVA test (n = 3), represented by values with different letters.
[3]Analysis was performed by MidWest Laboratories, Inc., Omaha, NE, USA.

Table 4: Response of juvenile yellowtail snapper (mean initial weight = 3.40 ± 0.06 g) fed diets containing 36% protein with varying lipid levels within a 10-week period for Trial 2. Values represent the means of three replicates.

Dietary Lipid	Final Biomass (g)	Final weight (g)	Weight Gain (g)	Weight Gain (%)	Total dry feed (g)	FCR[1]	FCE[2]	Survival (%)	TGC[3]	ANPR[4] (%)
7	161.28	18.81	7.44	446.30	19.42	2.62	78.88	85.00	0.03764	18.65 [ab]
10	151.08	18.79	6.89	455.03	18.83	2.76	81.67	80.00	0.03573	16.65 [b]
13	177.20	20.07	7.69	483.29	20.09	2.62	82.76	87.50	0.03850	18.62 [a]
16	136.28	15.90	6.30	392.79	17.62	2.84	71.56	85.00	0.03405	16.54 [ab]
PSE[5]	8.84	0.65	0.26	15.73	0.44	0.05	1.92	2.23	0.00	0.14
p-value[6]	0.4535	0.128	0.244	0.2354	0.2486	0.3569	0.1548	0.7273	0.3441	0.0254
Pro48: Lip10 Otohime EP3 commercial feed	202.70	20.90	9.37	499.62	22.38[a]	2.40	77.46	97.50	0.04396	15.42

[1]FCR = Feed conversion ratio = feed offered / (final weight-initial weight).
[2]FCE = Feed conversion efficiency = (final weight-initial weight) / feed offered.
[3]TGC = Thermal-unit growth coefficient.
[4]ANPR = Apparent net protein retention.
[5]PSE = Pooled standard error.
[6]One-way analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$). Tukey's multiple comparison test was used to determine statistically significant differences between treatment means when there was statistical significance in the ANOVA test (n = 3), represented by values with different letters.
[7]Commercial feed, Otohime EP3, 48% protein 10% lipid (Reed Mariculture).

Table 5: Whole-body composition (on wet weight basis) of yellowtail snapper fed a diet containing 36% protein with varying lipid levels for Trial 2.

Dietary Lipid	Moisture %	Protein (crude) %	Fat %	Ash %
7	69.50 [a]	17.90	7.86 [b]	4.41
10	68.48 [a,b]	17.23	9.12 [a,b]	4.09
13	66.83 [b]	17.80	9.34 [a,b]	4.66
16	66.75 [b]	17.28	10.66 [a]	4.66
PSE	0.37	0.17	0.34	0.11
p-value	0.0049	0.4188	0.012	0.2485
Pro48:Lip10 Otohime EP3 commercial feed	66.35	18.23	10.70	5.04

[1]PSE = Pooled standard error.
[2]One-way analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$). Tukey's multiple comparison test was used to determine statistically significant differences between treatment means when there was statistical significance in the ANOVA test (n = 3), represented by values with different letters.
[3]Analysis was performed by MidWest Laboratories, Inc., Omaha, NE, USA.

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CHAPTER V

EFFECTS OF SALINITY ON GROWTH, SURVIVAL, AND SERUM OSMOLALITY OF YELLOWTAIL SNAPPER, *Ocyurus chrysurus* (Bloch, 1971)

Abstract

The possible culture of high-value snapper species like the yellowtail snapper *Ocyurus chrysurus* in lower salinity conditions compared to full strength salinity oceanic water could promote the cultivation of this high-value species and open possible sustainable and more economic mariculture approaches. Two trials were conducted to determine the salinity tolerance of yellowtail snapper under varying salinity conditions. The first trial examined salinities of 3, 6, 9, 12, 16, 20, 26, and 32 g/L and evaluated serum osmolality within 24h, 48h, 72h, and 96h. The second trial evaluated growth performance and survival within a six-week culture period across a wide range of salinities of 6, 12, 16, and 32 g/L. The isosmotic point of yellowtail snapper was estimated to be 392.04 mmol/kg, which corresponds to 12 g/L salinity. Survival was significantly different ($p < 0.05$) for fish reared in 6 g/L, which may be due to increased osmoregulatory energy expenditure. Interestingly, significant differences ($p < 0.05$) were observed for biomass, with the highest weights observed in 12 g/L and 16 g/L. Such findings serve as initial data in the possible potential of red and yellowtail snapper culture in lower salinity conditions.

Key words: yellowtail snapper, serum osmolality, osmoregulation, salinity tolerance, isosmotic point, salinity, growth trial

1. Introduction

The yellowtail snapper *Ocyurus chrysurus* is distributed throughout the tropical and subtropical western Atlantic Ocean from the United States of America to Brazil (Gilmore, 1995; De la Morinière et al., 2002). This species has a high-market value and supports an economically important commercial and recreational fishing industry in the West Atlantic (McClellan and Cummings, 1998; Coniza et al., 2012). To date, studies on the culture of this low salinity tolerant species are limited.

Salinity is an environmental factor that frequently affects the physiology of aquatic organisms (Urbina and Glover, 2015). Changes and interference with physiological homeostasis and routine biological processes such as salinity changes can cause fish stress (Kültz, 2015), affecting activities like drinking rate (Evans et al., 2005) and osmoregulation (Fielder et al., 2007) to maintain body osmolality and ionic balance. Since salinity plays a big role in fish physiology, and is considered as one of the most primary environmental factors affecting aquatic habitats, salinity trials reveal their importance with regard to fish growth performance (Rubio et al., 2005). Previous studies have demonstrated that environmental salinity alters fish metabolic rate (Dutil et al., 1997), food intake (Rubio et al., 2005), digestive enzyme activity (Moutou et al., 2004) and feed conversion efficiency (Alava, 1998), which are closely related to growth performance of fish. Salinity tolerance is an important consideration in the culture of marine and freshwater organisms. It provides information about basic husbandry requirements necessary for the species to thrive in captivity as well as potential applications for the cultured organisms. In general, better growth of fish is observed when fish are cultured at intermediate salinities; however, the underlying mechanisms are still debatable (Bœuf and Payan, 2001; Moutou et al., 2004), making research studies into low salinity aquaculture of marine species an interesting topic for research.

While the impetus for the current dissertation is the evaluation of *O. chrysurus* as an aquaculture candidate; investigations into its salinity tolerance will provide valuable information about the species' ability to handle osmoregulatory stressors and could further be extrapolated for possible ecological applications. Understanding the response of yellowtail snapper to different salinities is important in assessing it's potential as an aquaculture species, as there is interest in culturing this species in RAS systems for which reduced salinities would have an economic advantage. Salinity tolerance is of considerable interest to commercial investment groups in developing affordable culture management since it would lower economic and maintenance costs.

The purpose of this study was to determine the salinity tolerance of *O. chrysurus*, a potential candidate for marine aquaculture. Two trials were conducted in this study. The first trial evaluated the lowest possible salinity level that yellowtail snapper could tolerate based on survival and serum osmolality, while the second trial involved a six-week growth period of yellowtail snapper under different salinity conditions. This investigation represents the first comprehensive study focused on the salinity tolerance of *O. chrysurus.*

2. Materials and Methods

2.1 Salinity tolerance trial

Yellowtail snapper used for both trials were obtained from wild brood-stock spawned in August 2021 at The Whitney Laboratory for Marine Bioscience at University of Florida. All experimental trials were conducted at E.W. Shell Fisheries Center at Auburn University in Auburn, Alabama where fish were held in an indoor recirculating system (Trial 1 with thirty-six 82.9-L glass rectangular tanks connected to a common reservoir tank (800-L); Trial 2 with twenty-four 730-L polyethylene circular tanks connected to a common reservoir tank (1600-L) with adequate

water quality parameters maintained within acceptable ranges for marine fish culture. Prior to the salinity trials, DO, temperature, salinity, pH, total ammonia nitrogen (TAN), and nitrite were maintained at 6.51 ± 0.06 mg/L, 26.34 ± 0.1 °C, 28.32 ± 0.16 g/L, 7.83 ± 0.03, 0.45 ± 0.06 mg/L, and 0.97 ± 0.19 mg/L respectively. Fish were fed a commercial feed for marine fish (crude protein 48%, crude lipid 12%, crude fiber 2%, crude ash 17%, Calcium 2.2%, Phosphorus 1.7%; Otohime EP3, Reed Mariculture) until the beginning of the experimental trials.

Two trials were conducted to determine the salinity tolerance of yellowtail snapper. The first trial was conducted to determine the lowest possible salinity level that yellowtail snapper could tolerate based on survival and serum osmolality. The salinity level treatments were 3, 6, 9, 12, 16, 20, 26, and 32 g/L. The second trial involved a six-week growth period of yellowtail snapper under different salinity conditions at 6, 12, 16, and 32 g/L. For Trial 1, the experiment was conducted in isolated 82.9-L glass rectangular tanks in triplicate with 10 juvenile fish (16.79 ± 1.94 g) per tank, with no significant difference among weights at stocking (p = 0.9355). Water salinity treatments were prepared two days prior to the onset of the experiment by mixing reconstituted sea salt (Crystal Sea Marinemix, Baltimore, MD, USA) with dechlorinated fresh water from Water Works Board of the City of Auburn (salinity 0.1 g/L, pH 7.3, alkalinity 29 ppm) to generate experimental solutions of 3, 6, 9, 12, 16, 20, 26, and 32 g/L. Fish were acclimated to the designated salinity treatments in 20 L buckets, either by dripping freshwater or the prepared experimental solution from experimental tanks into the bucket over time for low salinity and high salinity treatments respectively. A wide variety of salinity acclimation rates per hour have been used for snapper culture from 0.08 g/L for Pacific red snapper, *Lutjanus peru* (Castillo-Vargasmachuca et al., 2013), 0.21 g/L for gray snapper, *Lutjanus griseus* (Wuenschel et al., 2004), 0.42 g/L for schoolmaster snapper, *Lutjanus apodus*, (Trehern et al., 2020) to abrupt salinity

changes of 15 g/L for Australian snapper, *Pagrus auratus* (Fielder et al., 2007) to 24 g/L for mangrove red snapper, *Lutjanus argentimacultus* (Estudillo et al., 2000). For the present research, acclimation was accomplished over the course of several hours at a rate of 4 g/L salinity change per hour to provide enough time for greater equalization of ions between fish serum and the surrounding water acclimation medium. Fish were not fed during the experimental period. Right after the acclimation period, fish were introduced to individual static experimental tanks, equipped with their own miniature fluidized bed bio-filter and adequate aeration. Fish were not fed during the experimental period, and mortalities and swimming behavior were observed and recorded throughout the experiment. Fish mortalities (determined as no opercular movement and no response to prodding) were recorded and removed from the experimental tank daily.

2.2 Effect of salinity on fish serum osmolality

Blood was collected from three fish from each replicate salinity treatment at different time points 24h, 48h, 72h, and 96h after acclimation, for the determination of serum osmolality. Fish were euthanized with Tricane-S (MS-222 tricane methane sulfonate salt, Western Chemical, Inc., Ferndale, WA, USA). A non-heparinized 1-cc syringe was used for blood collection through the caudal vein of each fish and collected into non-heparinized Eppendorf tubes. Blood was immediately centrifuged (Fisher Scientific: Marathon 16km, USA) at 3000 rpm for 15 minutes to separate the clot from the serum. Total osmolality of serum and the water sampled from each tank were measured using 10 μL of sample by dewpoint depression using an osmometer (Wescor Vapro 5520 Vapor Pressure Osmometer, Logan, Utah).

2.3 Effect of salinity on fish growth performance

For Trial 2, the experiment was conducted in 730-L polyethylene circular tanks in triplicate with 20 juvenile fish (15.98 ± 0.30 g) per tank, with no significant difference among weights (p = 0.6485) at stocking. Water salinity treatments were prepared as previously described to generate experimental solutions of 6, 12, 16, and 32 g/L. Fish were acclimated to the designated salinity treatments in 20 L buckets, either by dripping freshwater or the prepared experimental solution from the experimental tanks into the bucket over time for low salinity and high salinity treatments respectively. Acclimation was accomplished over the course of several hours at a rate of 4 g/L salinity change per hour to provide sufficient time for greater equalization of ions between fish serum and the surrounding water acclimation medium. Immediately following the acclimation period, fish were introduced to individual static experimental tanks, each equipped with their own miniature fluidized bed bio-filter and adequate aeration.

The growth trial was conducted for six weeks in 6, 12, 16, and 32 g/L salinity treatments, with three replicate groups per salinity treatment randomly assigned. Fish were offered a commercially available feed for marine fish (crude protein 48%, crude lipid 12%, crude fiber 2%, crude ash 17%, Calcium 2.2%, Phosphorus 1.7%; Otohime EP3, Reed Mariculture) twice daily via two equal feedings. Fish were counted and weighed every other week to adjust the daily feed ration, which was calculated based on expected growth, body weight, and feed response. During this process, fish were dipped in a solution of chloroquine phosphate (MP Biomedicals, Solon, OH) at a concentration of 60 mg/L for around 2 min followed by a 10-15 s dip in unchlorinated freshwater, to minimize possible amyloodinium infections. At the end of the 6-week growth trial, fish were counted, and group weighed by replicate tank to determine mean final biomass, final weight, survival, percent weight gain, feed conversion ratio (FCR), and thermal-unit growth

coefficient (TGC). Fish were packed in sealed bags and stored in a freezer (-20°C) for proximate analysis.

2.4 Water Analysis

Dissolved oxygen was maintained near saturation using air stones in each culture tank. During both trials, all water quality parameters were taken in each static tank where dissolved oxygen, salinity, and water temperature were measured twice daily using a YSI-55 multiparameter instrument (YSI corporation, Yellow Springs, Ohio, USA), and total ammonia N (TAN) and nitrite-N were measured twice per week using a YSI 9300 photometer (YSI, Yellow Springs, OH). The pH of the water was measured twice weekly during the experimental period using a pHTestr30 (Oakton Instrument, Vernon Hills, IL, USA). All water quality parameters for Trial 1 and Trial 2 are presented in Table 1 and 2, respectively.

2.5 Statistical Analysis

All data were analyzed using SAS (V9.4, SAS Institute, Cary, NC, USA). Growth indices were analyzed using one-way ANOVA to determine significant differences ($P < 0.05$) among treatments followed by Tukey's multiple comparison test to evaluate significant differences between treatment means.

3. Results

3.1 Serum osmolality of yellowtail snapper in different salinities

Serum osmolality of yellowtail snapper *O. chrysurus* after 24h under different salinities are presented in Table 3. Significant differences were observed for serum osmolality, water osmolality, and osmoregulatory capacity for all treatments. The lowest serum osmolality ($P < 0.05$) was observed in fish acclimated to 3 g/L salinity (273.00 ± 2.19), while no significant differences ($P > 0.05$) were observed for all remaining salinity treatments. Significant differences ($P < 0.05$)

were also observed for osmoregulatory capacity across all experimental salinity treatments, with the lowest osmoregulatory capacity observed in yellowtail snapper acclimated to 12 g/L, while the highest was observed with fish acclimated to 26 g/L which was not different from those acclimated at 32 g/L. Serum osmolality of yellowtail snapper and rearing medium osmolality after 24h acclimation to different salinities is presented in Figure 1 to depict the level of fish osmoregulation. When comparing the slope of the curve for blood osmolality against the water osmolality, serum changes were relatively small compared to the increase in water osmolality, which was at 0.21 and 0.94, respectively (Figure 1). Figure 1 depicts that the fish were capable of maintaining the osmolality of blood serum stable when the salinity of the rearing medium was around 12 g/L and above. Below ~6 g/L salinity, serum osmolality decreased in a manner that approximated osmoconformity. The isosmotic point of yellowtail snapper was estimated to be 392.04 mmol/kg, which corresponds to 12 g/L salinity (intersection of two lines in Figure 1).

Fish survival acclimated to different salinities 3, 6, 9, 12, 16, 20, 26, and 32 g/L through a time-series from 24h, 48h, 72h, and 96h are presented in Figure 2. Upon consideration of the time-series effect of salinity treatments, yellowtail snapper were not able to tolerate 3 g/L, as survival was 30% after 24h, and none survived following 36h. After 36h, fish acclimated to salinities of 6, 9, 12, 16, 20, 26, and 32 g/L had 70% survival, which further decreased after 72h and 96h.

3.2 Growth performance of yellowtail snapper in culture medium of different salinities

Significant differences ($P < 0.05$) were observed for final biomass and survival, having the same trend with the highest values demonstrated by fish acclimated at 12 and 16 g/L, and lowest values for yellowtail snapper acclimated at 6 g/L (Table 4). Additionally, fish acclimated at 6 g/L had the lowest weight gain%, final biomass, and final weight. Fish acclimated at 12 and 16 g/L

demonstrated similar values for final weight, FCR, as well as survival compared to yellowtail snapper acclimated at 6 g/L and 32 g/L. Fish acclimated at 12 and 16 g/L also ate more with higher total dry feed offered, followed by yellowtail snapper acclimated at 32 g/L and lastly fish acclimated at 6 g/L. Although not significantly different ($P > 0.05$), highest weight gain % and best FCR values were demonstrated by fish reared at 16 g/L.

4. Discussion

The current study assessed serum osmolality and survival of yellowtail snapper across a range of salinity treatments, with an emphasis on the first 24h, and evaluated growth performance over a six-week period at different salinity treatments. In teleost fish, blood osmolality ranges from 280–360 mmol kg^{-1}, and is tightly regulated in a species-dependent range of salinities (Varsamos et al., 2005). In the present study, yellowtail snapper serum osmolality ranged between 273.00 mmol $kg^{-1}$and 404.44 mmol kg^{-1}, in fish reared at salinities from 3 g/L to 32 g/L, respectively (Table 3). Blood osmolality was significantly greater from all salinity treatments other than fish acclimated at 3 g/L, indicative of the strong osmoregulatory capacity of the fish within the short-term trial. However, low survival was observed at acclimation, proving that yellowtail snapper were unable to maintain strong osmoregulatory capacity over a long-term culture period.

Salinity is due to the presence of salts dissolved in water, which represent 60 of the 92 'basic' chemical elements (Riley, 1965). Chloride Cl^- (560 mM) and sodium Na^+ (450 mM) are the most important in normal salinity sea water (SW, 35 psu, 1050 mOsm l^{-1}) (Bœuf and Payan, 2001). Water with low ionic concentration is likely to provide substantial loss of ions from fish to the external water medium (Fielder et al., 2001). Reduction in Australian snapper growth and FCR was attributed with low K^+ concentrations in saline groundwater (Fielder et al., 2001), which was similar to chinook salmon when fed with K^+ deficient diets (Shearer, 1988). Similarly, euryhaline

species such as red drum reduced growth and FCR when cultured in hypotonic water (Gatlin III et al., 1992). Significant growth reduction in red snapper, *Lutjanus campechanus* was observed when reared in 8 g/L salinity compared to the fish reared in 32 g/L salinity (Galkanda-Arachchige et al., 2021). Such reduced growth could be, at least partially, due to the increased osmoregulatory energy expenditure at lower salinities (Galkanda-Arachchige et al., 2021). In juvenile cobia, significant mortalities were observed when fish were subjected to salinity acclimation of 2 g/L day^{-1} at salinities below 8 g/L (Atwood et al., 2004). Similarly, growth reduction were also observed when cobia were reared in salinities of 5 and 15 g/L (Denson et al., 2003).

Fish stress associated with osmotic shock and/or confinement caused a general reduction in serum osmolality, Na^+, and Cl^- in juvenile Australian snapper, *Pagrus auratus* acclimated in ambient seawater (30 g/L) to concentrated hyperosmotic (45 g/L) and diluted hyperosmotic (15‰ g/L) environments (Fielder et al., 2007). A study on spotted tail goby, proved that ion concentration in the body fluids changes when fish were challenged by medium salinity fluctuations (Shui et al., 2018). Various studies have proven elevated levels of serum osmolality such as in spotted tail goby, where an increase in serum osmolality, Cl^-, Na^+ and K^+ were observed when fish were transferred in 40 and 50 salinity indicating that dehydration occurred due to the osmotic efflux of water from the body and excess influx of ions from the hyperosmotic environment (Hwang et al., 1989). Similarly, in a study with rabbitfish *Siganus rivulatus*, although similar growth was observed at all salinities tested, all other results focused on plasma osmolality and gill Na+ –K+ – ATPase activity (NKA) suggest that rabbitfish perform better at 35 ppt than at other salinities (Saoud et al., 2007).

However, reduction in serum osmolality, Cl^-, Na^+ and K^+ were also observed in spotted tail goby when fish were transferred to freshwater, indicating that osmotic influx occurred, in

accordance with dilution of serum ion concentration even redistribution of ions between serum and cells (Bath and Eddy, 1979). Lowered serum Na and osmolality could be an indication of osmoregulatory difficulties (Laiz-Carrión et al., 2005; Mylonas et al., 2009). A study on juvenile GIFT tilapia demonstrated significant decrease in plasma osmolality at salinities of 0‰ and 20‰, suggesting a weak osmoregulatory capacity to tolerate a wide range of salinities for juvenile GIFT tilapia (Qiang et al., 2013). Likewise, a study on white sturgeon showed a reduction in blood hematocrit and hemoglobin concentrations at higher salinities over the course of the 120h exposure through blood dilution, primarily caused by osmotically obliged water movement (Amiri et al., 2009).

Changes in salinity were also reported to have no effects on blood osmolality when tested on milkfish reared from 0 g/L to 35g/L (Lin et al., 2003) as well as for the Mozambique tilapia when either in freshwater or saltwater (Hwang et al., 1989). Likewise, no significant osmoregulatory disturbances in the salinity range of 5–50 g/L were observed on gray snapper, *Lutjanus griseus* when challenged with different salinity treatments and blood samples were collected at various time points post-transfer (Serrano et al., 2011). In contrast, gray snapper exposed at extreme salinities of 0 and 60 g/L, showed significant but transient changes in osmolality and/or hematocrit were observed (Serrano et al., 2011). Significant changes were observed on gilthead sea bream when reared at 12 g/L and 6 g/L but no difference when fish were reared at 12 g/L and 38 g/L (Laiz-Carrión et al., 2005). Such studies indicate there is a wide range of salinity change variation that different fish species can tolerate.

The present study strengthens the argument that euryhaline teleostan fish tend to be strong osmoregulators. When comparing the slope of the curve for blood osmolality against the water osmolality, serum changes are relatively smaller as compared to the immense increase in water

osmolality, which was at 0.21 mmol kg^{-1} and 0.94 mmol kg^{-1} respectively (Figure 1). A study on flounder yielded similar results where salinity affected plasma osmolality very minimally, probably due to the fact that flounder were already adapted to face the salinities it osmoregulated in to (Sampaio and Bianchini, 2002). A study on evaluating the effect of abrupt transfer of juvenile Australian snapper, *Pagrus auratus* from ambient seawater (30‰) to concentrated hyperosmotic (45‰) and diluted hyperosmotic (15‰) environments demonstrated that snapper can osmoregulate in a wide range of salinity and provide indirect evidence that both filament and lamellar chloride cells are responsible for excretion of excess salt from snapper in hyperosmotic environments (Fielder et al., 2007).

In the present study, significant differences were observed in the osmoregulatory capacity of yellowtail snapper, wherein the lowest values were observed at 12 g/L and 16 g/L (which were not significantly different from each other), suggesting that lesser energy is utilized in maintaining ionic balance at said rearing medium salinity levels which refers to the isosmotic point (Figure 1). The isosmotic point for this study is determined at 392.40 mmol kg^{-1}, which corresponds to 12 g/L salinity (Figure 1). Similar results were demonstrated by a study in hybrid tilapia (*Oroechromis mossambicus* ♀ *O. hornorum* ♂), where the lowest osmoregulation costs were found at an isoosmotic salinity of 12% (Febry and Lutz, 1987; Suresh and Lin, 1992; Tran-Ngoc et al., 2017), and higher osmoregulation energy costs when reared in freshwater. Such studies showed that less energy is required to maintain the ion balance in the salinity of 15% for hybrid tilapia, with the energy being directed towards nutritional digestion activity.

After 24h acclimation, no mortalities were observed for any surviving salinity treatments except for yellowtail snapper acclimated to 3 g/L (Figure 2). The low survival of fish acclimated at 3 g/L may be attributed to the significantly lower serum osmolality at 273.00±2.19 mmol kg^{-1}

(Table 3). In a study by (Fielder et al., 2005), salinity was proven to have a significant effect on survival, growth, and development of Australian snapper larvae. All larvae held in 5 g/L died within 48 h of transfer from 35 g/L to 5 g/L, while some larvae survived for 18 days in all salinities from 10 g/L to 45 g/L (Fielder et al., 2005). Significant survival and growth reduction in snapper larvae at 45 g/L was associated with the energy cost of osmoregulation (Fielder et al., 2005). This has implications for reduced productivity of snapper in hatcheries located in areas with high salinity water intake.

The isosmotic value for Australian snapper and red sea bream is approximately 12g/L (Woo and Fung, 1981; Fielder et al., 2008), which is in agreement to the results of the present study (Figure 1). Results of the present study (Trial 1) show that all salinity level treatments had 70% survival after 36h, which can be attributed to handling stress for blood sampling. Trial 1 results, as proven by survival and serum osmolality data show that yellowtail snapper was able to tolerate salinity level treatments as low as 6 g/L.

After determining the level of salinity tolerance, the possibility of rearing the yellowtail snapper in a longer growth trial under such low salinity was considered. Salinity level treatments for Trial 2 were determined by using the results of Trial 1 as basis. After the 6-week growth trial, no significant differences ($P > 0.05$) were observed in the survival of yellowtail snapper acclimated from 12 g/L, 16 g/L, and 32 g/L (Table 4). Such surprising results serve as evidence that 96h acclimation period (based on the results of Trial 1), are useful indicators of a species' ability to survive long term in selected treatment conditions (DiMaggio et al., 2009).

Salinity is known to have a greater effect on plasma osmolality and Na+, K+- ATPase activity than water temperature (Qiang et al., 2013). Longer acclimation though, can affect such serum and ionic changes by returning back to original levels or was higher than the initial levels

(Shui et al., 2018). Effects of salinity on fish growth vary greatly among fish and among salinity ranges tested (Bœuf and Payan, 2001). Aside from this, osmoregulation is another factor to consider, as it requires increased expenditure of metabolic energy, which may result in reduced fish performance (Bryan et al., 1988; Fielder et al., 2001). A study demonstrated reduction in the size (filament and lamellar) and number (filament) of chloride cells in the yellowtail or Japanese amberjack, *Seriola quinqueradiata* when transferred from 30 g/L to 15 g/L (Sala et al., 1987). This reduction in lamellar chloride cells reflects its reduced need to actively excrete Na^+ and Cl^- in the diluted hyperosmotic environment (Fielder et al., 2007).

Results of Trial 2 for the present study depicts that yellowtail snapper acclimated in 12 g/L and 16 g/L exhibited highest weight gain %, best FCR values, and highest survival rate as opposed to fish acclimated at 6 g/L and 32 g/L (Table 4). Such findings prove the effect of long-term salinity acclimation exposure on growth performance, feed utilization, and survival of yellowtail snapper. A similar study on red snapper demonstrated no significant differences for the survival and FCR when fish were reared in 8 and 32 g/L salinities (Galkanda-Arachchige et al., 2021). In addition, a study on gilthead sea bream, *Sparus aurata* larvae showed that survival and wet and dry weights of 32 dah were greatest when salinity was reduced from 40 g/L to 25 g/L (Tandler et al., 1995). Interestingly, the energetic response of juvenile gray snapper to temperature and salinity proved that snapper acclimated at lower salinities required lesser food to achieve the same somatic growth as juveniles in high salinity habitats (Wuenschel et al., 2004). Although salinity effects appear small, it has considerable impact on growth when integrated over the entire (~60–120 days) juvenile nursery period (Wuenschel et al., 2004).

Although feed composition was not considered a variable in this trial, some studies have proven the effect of salinity on feed utilization and digestibility in fish. Amino acid levels in tissues

of some teleost fish have been proven to increase when exposed to elevated water salinities (Jarvis et al., 2001). However, other studies report salinity to be independent from total nutrient absorption (Nordrum et al., 2000). The influences of salinity on measured rates of amino acid absorption are more complex to understand because they can involve changes in the carrier-mediated and carrier-independent pathways of influx (Nordrum et al., 2000).

5. Conclusion

In summary, the present study shows that changes in salinity levels elicit changes in serum osmolality and survival in yellowtail snapper, occurring mostly during the first 24h of acclimation. Yellowtail snapper were able to tolerate salinity levels as low as 6 g/L after 96h. The 6-week growth trial clearly indicates that yellowtail snapper is capable of tolerating salinity levels that are lower than seawater for an extended period of time. The isosmotic point of yellowtail snapper was estimated to be 392.04 mmol kg^{-1}, which is corresponds to 12 g/L salinity. Growth performance and survival data substantiate the potential of *O. chrysurus* as a possible candidate for marine finfish aquaculture. Survival was significantly different for fish reared in 6 g/L, which may be due to increased osmoregulatory energy expenditure. Interestingly, significant differences were observed for biomass, with highest weights in 12 g/L and 16 g/L. Understanding the physiological capacity of yellowtail snapper to adapt to environment salinity changes is essential information for planning sites and culture protocols for aquaculture of this species. Our results support the idea that the yellowtail snapper is suitable for aquaculture in estuaries where rapid fluctuations in salinity occur, or in low-salinity waters. Therefore, based on the results of the current study, the salinity of 12 g/L to 16 g/L is proposed an effective working salinity for future research studies such as temperature tolerance, stocking density, and diet studies.

Table 1: Summary of water quality parameters recorded during the 92h salinity tolerance trial conducted to evaluate serum osmolality of yellowtail snapper (mean weight = 16.79 ± 1.94 g) in different salinities for Trial 1. Values represent mean ± SE of three replicates.

Parameter	3 g/L	6 g/L	9 g/L	12 g/L	16 g/L	20 g/L	26 g/L	32 g/L
DO	7.54±0.14	7.06±0.09	7.20±0.56	6.77±0.18	6.71±0.14	7.17±0.17	6.61±0.13	6.41±0.17
Temperature	23.39±0.25	27.79±0.29	25.06±5.13	26.09±0.25	25.86±0.64	23.63±0.66	25.33±0.60	23.87±0.64
Salinity	3.19±0.07	6.32±0.03	9.43±0.05	12.41±0.05	16.31±0.07	20.73±0.09	26.37±0.06	32.47±0.10
Alkalinity	0.00	0.00	0.00	28.33±8.82	20.00±8.66	55.00±22.55	96.67±441	121.67±20.28
pH	7.50±1.66	7.39±0.07	7.29±0.11	7.09±0.19	7.66±0.06	7.73±0.04	7.66±0.03	7.45±0.09
TAN	0.93±0.29	0.90±0.25	2.13±0.20	1.90±0.70	1.18±0.21	0.50±0.17	1.93±0.55	3.50±1.53
Nitrite	1.01±0.27	1.67±0.84	1.38±0.12	2.38±0.12	0.72±0.10	1.22±0.09	1.73±0.42	1.43±0.45

Table 2: Summary of water quality parameters recorded during the trial conducted to evaluate growth parameters of yellowtail snapper (mean weight = 15.98 ± 0.30 g) in different salinities for Trial 2. Values represent the mean ± SE of three replicates.

Parameter	6 g/L	12 g/L	16 g/L	32 g/L	PSE[1]	p value[2]
DO	7.88±0.04[a]	7.80±0.04 [a]	7.38±0.04 [b]	6.84±0.04 [c]	0.0241	< 0.0001
Temperature	25.61±0.06	25.82±0.92	25.41±0.08	25.68±0.10	0.2321	0.9383
Salinity	5.98±0.03 [d]	12.09±0.04 [c]	16.39±0.11 [b]	32.62±0.11 [a]	0.2961	< 0.0001
pH	6.86±0.08 [a]	6.37±0.08 [b]	6.23±0.07 [b]	6.81±0.10 [a]	0.0471	< 0.0001
TAN	1.71±0.34	2.72±0.39	3.96±0.55	6.51±2.29	0.6159	0.0349
Nitrite	0.20±0.02 [b]	0.25±0.07 [ab]	0.58±0.16 [ab]	1.53±0.29 [a]	0.0944	< 0.0001

[1]PSE = Pooled standard error.

[2] One-way analysis of variance and Tukey post-hoc test was used to determine significant differences ($p < 0.05$) among treatments (n = 3), represented by values with different letters.

Table 3: Serum osmolality (mmol/kg) of yellowtail snapper (mean weight = 16.79 ± 1.94 g), after 24 hours of acclimation to different salinities in Trial 1. Values represent the mean ± SE of three replicates.

Salinity (g/L)	Osmolality in serum	Osmolality in water	Osmoregulatory Capacity
3	273.00±2.19^{b}	134.89±4.55^{e}	138.11±5.83^{cd}
6	392.78±5.20^{a}	194.00±2.55^{de}	198.78±6.83^{bc}
9	401.00±13.30^{a}	288.33±5.54^{d}	112.66±18.10^{cd}
12	376.67±2.34^{a}	452.11±20.61^{c}	75.55±18.67^{d}
16	404.44±5.97^{a}	492.22±8.85^{c}	87.78±8.70^{d}
20	385.22±0.662^{a}	641.11±2.70^{b}	255.89±2.23^{b}
26	397.11±13.13^{a}	888.11±40.10^{a}	491.00±35.57^{a}
32	399.11±8.95^{a}	841.00±47.14^{a}	441.89±38.54^{a}
PSE[1]	1.96	27.38	19.75
p-value[2]	< 0.001	< 0.001	< 0.001

[1]PSE = Pooled standard error.

[2] One-way analysis of variance and Tukey post-hoc test was used to determine significant differences ($p < 0.05$) among treatments (n = 3), represented by values with different letters.

Figure 1: Serum osmolality (n=3) from yellowtail snapper *Ocyurus chrysurus* (16.79 ± 1.94 g), after 24 hours of acclimation to different salinities in Trial 1. The isoosmotic point was determined to be 392.40 mmol/kg, which corresponds to 12 g/L salinity.

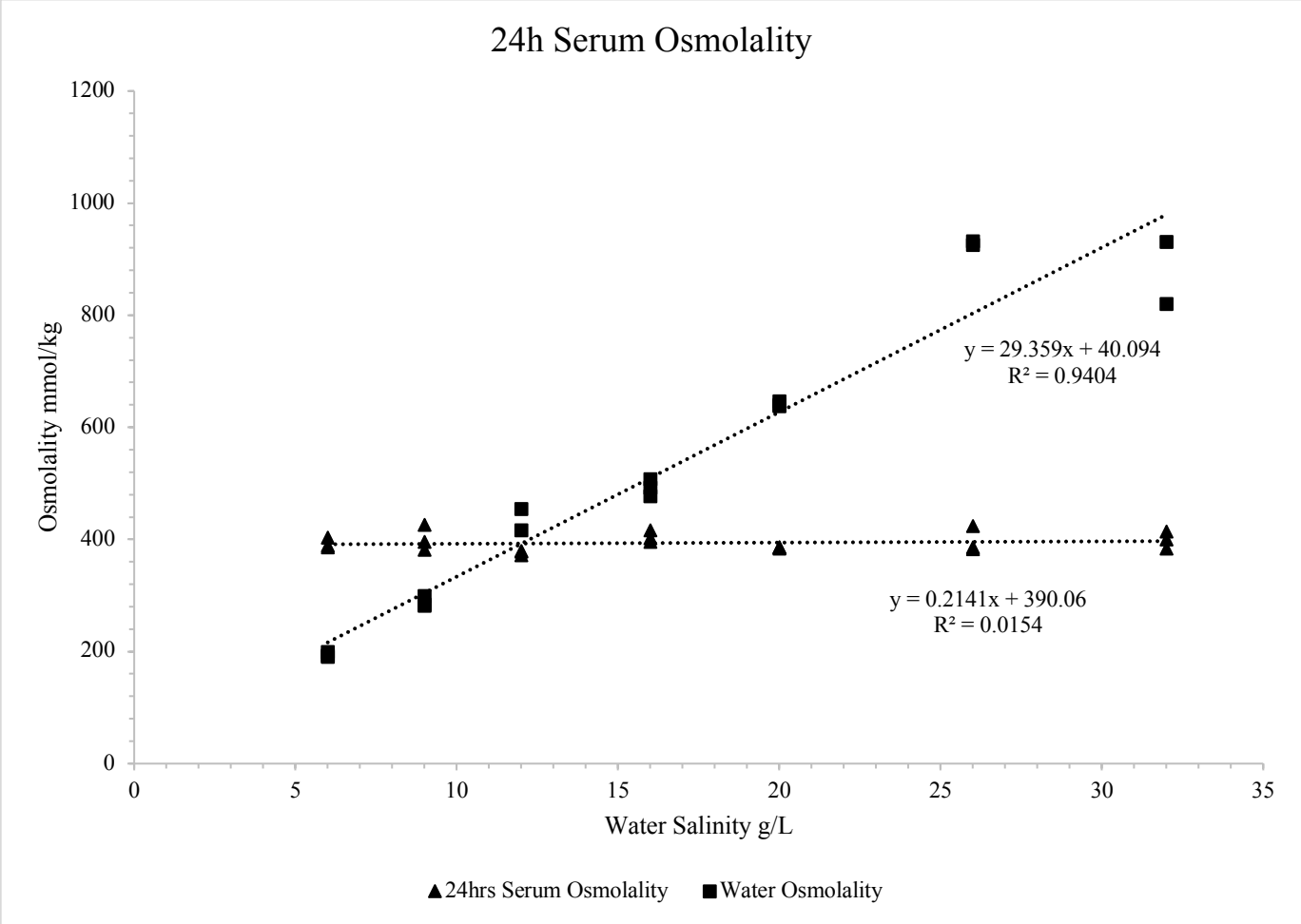

Figure 2: Survival (n=3) of yellowtail snapper *Ocyurus chrysurus* (16.79 ± 1.94 g), after 24 hours acclimation to different salinities.

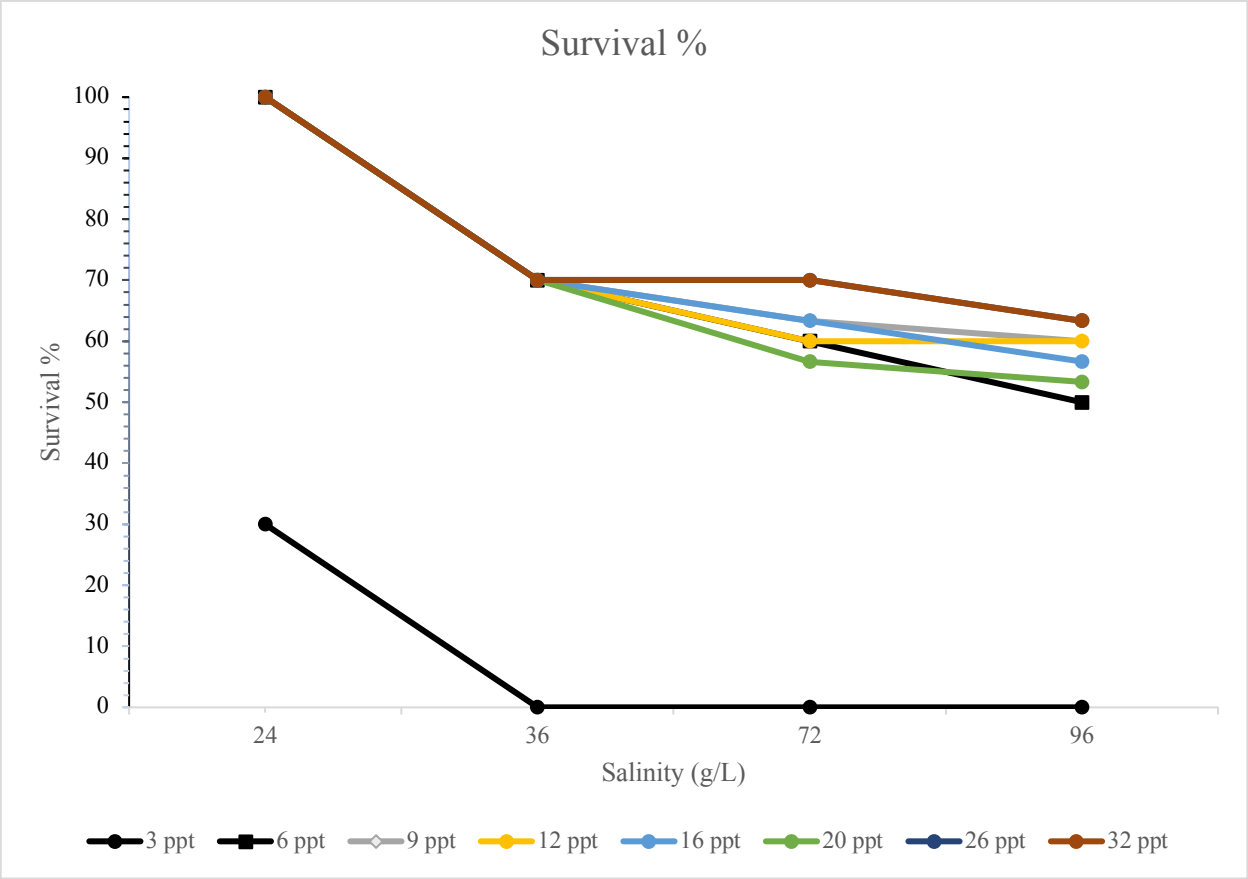

Table 4: Response of juvenile yellowtail snapper (mean initial weight = 15.98 ± 0.30 g) fed diets containing varying protein and lipid ration levels during a 6-week culture period. Values represent the means of three replicates.

Salinity (g/L)	Final Biomass (g)	Final weight (g)	Weight Gain (%)	Total dry feed (g)	FCR	Survival (%)
6 g/L	223.17 [b]	25.30	57.11	25.72±1.61	2.96±0.41	45.00[b]
12 g/L	600.40 [a]	34.57	107.62	30.62±1.85	1.73±0.10	88.33[a]
16 g/L	616.87 [a]	34.99	123.16	30.52±1.46	1.61±0.12	88.33[a]
32 g/L	508.00 [a,b]	28.58	83.38	26.74±2.94	3.57±1.94	86.67[a]
PSE[1]	120.06	5.97	33.64	3.55	1.72	14.14
p-value[2]	0.013	0.2122	0.1643	0.2771	0.4716	0.0132

[1]PSE = Pooled standard error.
[2]One-way analysis of variance and Tukey post-hoc test was used to determine significant differences ($p < 0.05$) among treatments (n = 3), represented by values with different letters.

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CHAPTER VI

SUMMARY AND CONCLUSION

Interest in the possible culture of high-value marine organisms is one strategy to address sustainable and more economical mariculture. The current research was designed to conduct nutritional studies on two high-value marine species namely the Pacific white shrimp, *Litopenaus vannamei*, and yellowtail snapper, *Ocyurus chrysurus.* Dietary Met requirement of Pacific white shrimp, *Litopenaus vannamei*, was determined by using three different Met sources (DL-Met, Met-Met, and Corn protein concentrate) through the analysis of growth performance, nutrient retention of a dose-response trial using a broken line model. Over-all growth performance significantly improved as Met supplementation increased, as verified by two experimental trials. Shrimp fed Met-Met supplemented diets performed better than DL-Met fed shrimp, specifically for Weight Gain %, FCR, and TGC. Nutrient retention values for protein and lysine significantly increased as Met supplementation increased, while Met retention significantly decreased as Met supplementation increased. The utilization of CPC as an intact protein source showed a dose response in all growth performance parameters and nutrient retention values and demonstrated better results than CAA supplementation. The present study determined the optimal dietary methionine requirement of *L. vannamei*, estimated by a one-slope broken-line regression analysis model. Based on weight gain%, the methionine requirement ranged between was 0.56 to 0.67% of the dry diet (corresponding to 1.55-1.84% of dietary protein on a dry-weight basis), and between 0.6053 to 0.6559% of the dry diet (corresponding to 1.68-1.82% of dietary protein on a dry-weight basis) based on TGC (Thermal Growth unit Coefficient). Such findings are crucial in formulating

cost-effective practical diets and utilizing intact proteins or purified CAAs for juvenile *L. vannamei*.

The potential of the yellowtail snapper *Ocyurus chrysurus* (Bloch, 1791), and its culture poses a promising reception and positive potential for development in the aquaculture industry. The present study evaluated the growth response of yellowtail snapper using experimental diets with different protein sources namely fishmeal, poultry meal, and soybean meal. Results from this study clearly indicate that fishmeal is the best protein source for yellowtail snapper practical diets as opposed to poultry meal and soybean meal. Significant differences were observed in all fish growth parameters in favor of the use of high fishmeal diets. Additionally, no significant intestinal morphological changes were observed in the intestinal histology morphometric measurements and scoring for yellowtail snapper fed 40% soybean meal inclusion, demonstrating that high soybean meal inclusion diets did not induce SBMIE in yellowtail snapper under the experimental conditions of this study.

Determination of the optimal protein and lipid concentration in experimental diets for yellowtail snapper was also evaluated. Varying levels of protein and lipid were compared to currently used commercial feed with high protein and lipid content. Results of Trial 1 showed that yellowtail snapper was able to effectively utilize practical diets with Pro36:Lip10 and Pro40:Lip10, as evidenced by growth performance and feed utilization parameters. Pro36:Lip10 with the lowest protein level among all treatments showed the highest ANPR% and survival. With exclusively considering the experimental diets in Trial 2 (not considering the commercial diet), yellowtail snapper fed Pro36:Lip13 had the best over-all performance.

The current study establishes baseline data as initial dietary information for yellowtail snapper nutrition and recommends a lower concentration of dietary protein within the range of

36%-40% and dietary lipid levels of 7%-13%, which are lower than the currently used commercial diets for marine finfish. The knowledge gathered from the current study may be helpful for nutritionists in formulating feed based on more sustainable, effective, and cheaper feedstuffs and promote sustainable yellowtail snapper aquaculture. The effects of varying salinity levels were proven to elicit changes in serum osmolality and survival in yellowtail snapper, occurring mostly during the first 24h of acclimation.

Yellowtail snapper were able to tolerate salinity levels lower than seawater, the lowest at 6 g/L, and for an extended period of time. Growth performance and survival data substantiate the potential of *O. chrysurus* as a possible candidate for marine finfish aquaculture. Interestingly, significant differences were observed for biomass, with highest weights in 12 g/L and 16 g/L. Understanding the physiological capacity of yellowtail snapper to adapt to environment salinity changes is essential information for planning sites and culture protocols for aquaculture. Our results support the idea that the yellowtail snapper is suitable for aquaculture in estuaries where rapid fluctuation in salinity occurs, or perhaps in regions where low salinity culture water is available. Therefore, based on the results of the current study, salinities within the range of 12 g/L to 16 g/L are proposed as a starting point for future research and as a salinity choice in experiments to study other environmental tolerances such as temperature, stocking density, diet etc. in the possible culture of the yellowtail snapper.

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