NUTRITIONAL STRATEGIES TO IMPROVE THE GROUPER AQUACULTURE INDUSTRY

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ADC : Apparent digestibility coefficient

BW : Body Weight

CP : Crude Protein

Cys : Cysteine

DE : Digestible Energy

DO : Dissolved Oxygen

DP : Digestible Protein

DPI : Department of Primary Industries

DP:DE : Ratio of g DP MJ DE^{-1}

ERE : Energy Retention Efficiency

FBW : Final Body Weight

FCR : Food Conversion Ratio

FM : Fishmeal

FO : Fish oil

GE : Gross Energy

GMBW: Geometric Mean Body Weight

His : Histidine

HIS : Hepatosomatic Index

IBW : Initial Body Weight

JCU : James Cook University

Lys : Lysine

MBW : Metabolic body weight

Met : Methionine

MJ : Megajoule of Energy

NFE : Nitrogen Free Extract

NSW : New South Wales, Australia

PRE : Protein Retention Efficiency

PSFI : Port Stephens Fisheries Institute

QLD : Queensland, Australia

RE : Retained Energy

SBM : Soybean Meal

SPC : Soy Protein Concentrate

Tau : Taurine

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1. Non-Technical Summary

This report presents a series of studies initially funded by the United Soybean Board to develop soy based aquafeeds for grouper and to investigate ways to optimize aquaculture management of grouper through optimizing feeding frequency and stocking density. The species focused on in this report are tiger grouper *Epinephelus fuscoguttatus* and gold spot grouper *E. coioides*.

A nutritional model was developed estimating the requirements for digestible protein (DP) and digestible energy (DE) for tiger grouper growing from approximately 10 g to 1 kg. A series of practical diet formulations are suggested based on the predicted DP and DE requirements for the grow out of tiger grouper to 1kg body weight considering three key growth stanzas; <100 g, 100 – 500 g and 500 – 1000 g. The diets utilize soybean meal (SBM) and soy protein concentrate (SPC) and different quality fishmeal's with either a 55% or 65% crude protein (CP) content.

Two feeding trials were conducted with gold spot grouper to identify an optimal SBM or SPC inclusion in diets. The first trial examined diets formulated with 10, 20, 30 or 40% SBM or SPC and also a diet containing 30% SBM and 20% brewer's yeast. Gold spot grouper were 84 g initial body weight (IBW). The results identified that good growth can be achieved with diets containing up to 30% SBM or 30% SPC and saw no difference in growth when compared to a fishmeal control diet. Gold spot grouper approximately tripled in body weight after eight weeks on all diets except for the 40% inclusion of both the SBM and SPC diets. Feed conversion ratio (FCR) was excellent at <1:1 for all diets up to 30% SBM or SPC inclusion. Grouper fed diets containing 40% SBM or SPC were the worst performing. Thresholds for SBM and SPC inclusion were estimated at 30.8% and 28.9% respectively using regression analysis. Histological analysis of the hind gut and pyloric caeca revealed no obvious negative effect of either SBM or SPC diets at any inclusion level after eight weeks feeding; however, there was a strong negative relationship with hepatosomatic index (liver size relative to body weight) and hepatocyte (liver cell) size to inclusion level. While growth was good using diets with up to 30% SBM or SPC, health impacts should be investigated for longer term feeding trials. Gold spot grouper fed the 30% SBM and 20% brewer's yeast ate more and were significantly larger than grouper fed the other diets after eight weeks indicating good potential for cost effective diet formulation with these ingredients; however, longer term assessment of this type of diet is required before recommendations for long term feeding can be made.

The second feed trial with gold spot grouper evaluated diets with different quality fishmeal, a premium fishmeal (75% CP) or a lower quality fishmeal (65% CP) of reclaimed fish trimmings, and a combination of SBM and SPC at 1:1 with a total inclusion of 30% based on the results of the first study. This trial used 500 g fish growing to >900 g over eight weeks. Grouper growth, survival and FCR's were excellent across all diet treatments and no differences were found among grouper fed any of the diet treatments including the fishmeal controls. Taken in combination with the results of the first trial, there is good potential for flexibility in diet formulation for gold spot grouper provided that diets are nutritionally balanced.

The final series of studies identified the optimal feed frequency and stocking densities for gold spot grouper reared in 200 L cages. The feed frequency trial considered four satiated feeding treatments; a single 0800 feed, a single 1600 feed, two feeds per day (0800 and 1600) or three feeds per day (0800, 1200, 1600). Grouper were stocked at 15 g IBW and grew to between 43 - 63 g depending on the feed frequency treatment. Fish fed the single morning feed performed the worst while fish fed the single afternoon feed and three feeds per day regime were the best performing with respect to growth. All FCR's were <1:1 with the fish fed a single afternoon feed having the best FCR of 0.83:1. The results of this study have clear implications for the feed management of gold spot grouper; a single afternoon feed is sufficient to elicit a good growth and feed conversion response. A single afternoon feeding regime can potentially reduce labour and feed costs associated with feeding and feed management of gold spot grouper.

The stocking density trial assessed the performance of gold spot grouper stocked at one of four different densities; 6, 15, 30 or 45 kg m^{-3}, growing from 300 g IBW to approximately 500-600 g depending on density treatment over eight weeks. Grouper stocked at the lowest density performed poorly compared to those stocked at densities of 15 kg m^{-3} or above where there was no difference in growth or FCR. FCR was approximately 1:1 at the lowest density and <1:1 at 15 kg m^{-3} or above. The results of this study demonstrate that gold spot grouper can tolerate high stocking densities while still maintaining good growth rates and feed efficiencies. Fish at the lowest density were observed to be skittish and reluctant to feed. This has practical implications for farm management practices as well as consideration for appropriate numbers of fish to use when running feed trials with this species if optimal growth rates are desired.

2. Background

Grouper area high-value marine fish species and aquaculture production of several grouper species is rapidly expanding throughout the Asia Pacific region. Most grouper farms continue to feed using low-value trash fish with extremely poor feed conversion efficiencies. Estimates of FCRs of trash fish for grouper range from 7.2 – 15.0 (De Silva and Turchini, 2009); by comparison FCR's for gold spot grouper fed compound feeds were <1.0 in laboratory trials and approximately 1.2 for humpback grouper fed commercial compound feeds during on-farm tank based trials (pers. obs.).

While the predominant feed used for grouper culture in SE Asia is still trash fish, commercial aquafeeds that are being used are often generic "marine fish" formulations with dietary specification which are often too high in dietary fat, resulting in compromised growth, high visceral fat deposition in the carcass and fatty liver pathologies. Commercial grouper aquaculture expansion cannot be sustained indefinitely unless there is a shift to the use of species-specific formulated feeds. One of the stumbling blocks to achieving this transition is a lack of scientific information and technical assistance to persuade farmers that it is in their immediate and long-term business interests to switch to formulated feeds.

To make the transition from feeding trash fish to formulated feeds requires an understanding of the nutritional requirements of the species. This information will then provide a platform from which the development of aquafeeds for grouper using fishmeal replacement proteins including soybean meal (SBM) and soy protein concentrate (SPC) can be made. To address these issues a series of projects were successfully conducted for the United Soybean Board (USB) to elucidate the nutritional requirements of tiger grouper (*Epinephelus fuscoguttatus*), and gold spot grouper (*E. coioides*) and to test the optimal inclusion level of SBM and SPC and fishmeal quality in diets for grouper and to also provide insight into feeding and aquaculture management through feeding frequency and stocking density studies respectively. This report collates key information from these studies to provide a synopsis of nutritional and aquaculture management strategies for grouper. In addition, this report also presents new information on the histological examination of grouper fed a range of SBM and SPC diets. From this information feed formulations are suggested with an emphasis on practical ingredients including SBM and SPC.

3. A Note on the Presentation of Feed and Ingredient Data: Dry Matter Vs As Is Basis

Unless otherwise indicated the feed ingredient and diet composition data in this report are presented on a Dry Matter basis, i.e. excluding the moisture content. This was done to standardize the presentation of the data and facilitate direct comparison among ingredients, diets or experiment treatments, within and across studies.

Terms such as As Is, As Received, As Fed and Wet Weight Basis all refer to the whole compound inclusive of moisture content. The moisture content of feeds and ingredients will naturally vary depending on the relative humidity and room temperature, effectively *diluting* or *concentrating* the nutrient content at a higher or a lower moisture content, respectively. Compositional data from analytical laboratories are often, but not always, reported on an As Received basis and formulators or anyone wishing to make aquafeeds should be aware of the form of the data that they are working with if precise diet specifications are required. Similarly, expressing feed intake on a dry matter basis is a more biologically accurate way of quantifying nutrient intake and nutrient conversion efficiencies, while gross feed weight may be more relevant for economic calculations.

Converting from an as is basis to a Dry Matter basis, or *vice versa*, for any nutrient is a straightforward process. The following examples use the soybean meal ingredient presented in Table 4.1.

Converting Dry Matter to as is basis:
Dry Matter (DM) content = 87.6%
Crude Protein (CP) content of ingredient at 100% DM = 54.0%

$$\frac{54.0\%\ CP}{100\%\ DM} = \frac{X\%\ CP}{87.6\%\ DM}$$

Solving for X

$$100X = 54.0 \times 87.6$$

$X = 47.3\%$ CP As Is

Converting as is to Dry Matter basis:
DM content = 87.6%
CP content of ingredient As Is = 47.3%

$$\frac{47.3\%\ CP}{87.6\%\ DM} = \frac{X\%\ CP}{100\%\ DM}$$

Solving for X

$$87.6X = 47.3 \times 100$$

$$X = 54.0\%\ CP\ DM$$

4. Research Trials

The following presents an overview of the key studies that were conducted for the United Soybean Board 2016-2018 on tiger and gold spot grouper. These studies set out to:

i) Develop a nutritional model quantifying dietary protein and energy requirements for tiger grouper.

ii) Determine digestibility coefficients of SBM and SPC diets and ingredients for tiger grouper
iii) Determine the optimal inclusion level of SBM and SPC in aquafeeds for gold spot grouper
iv) Assess fishmeal quality interactions in aquafeeds for gold spot grouper
v) Identify the optimal feeding frequency for gold spot grouper
vi) Determine the optimal stocking density for gold spot grouper

The key protein sources used to formulate feeds in this report were chosen as those that would be relevant for S.E. Asia. At the time of conducting this research the soy products that were available in Australia to manufacture the experiment diets were SBM of Argentinian origin and SPC of European Union origin. U.S soy was not being purchased in Australia at the time of conducting the research in this report and quarantine laws precluded the timely importation and use of U.S. soy.

4.1. Development of a Nutritional Model for Tiger Grouper

4.1.1. Introduction

Tiger grouper are a valuable commercial aquaculture species yet relatively little information is available that adequately describes their basic nutritional requirements for protein and energy, particularly for fish larger than 200 g. The main objective of this study was to better understand their nutritional requirements with this information providing a platform to improve feed management and feed formulation. A series of integrated studies were carried out to determine the requirements for digestible protein (DP) and digestible energy (DE) for maintenance and growth of tiger grouper. A bioenergetic factorial method (Booth, et al., 2010; Lupatsch and Kissil, 2005; Pirozzi, et al., 2010a) was utilized for this approach which provided a comprehensive assessment of the changing nutrient demand of the animal throughout grow-out production.

4.1.2. Materials and Methods

The factorial approach to developing a bioenergetic model for tiger grouper broadly consisted of several empirical studies examining the following themes:

i) Nutrient and energy utilization efficiencies
ii) Maintenance requirements for DP and DE
iii) The development of a growth model collated from laboratory data and literature values
iv) Whole body composition of tiger grouper ranging from <10 g to approximately 1 kg in body weight
v) Nutrient digestibility

Total Nutrient Requirement was modelled from the data sets derived from the above factors as:

$$a \times BW(kg)^{b} + c \times Growth \qquad \text{Eq.1}$$

where; a = maintenance requirement, BW = body weight (g), b = body weight exponent, c = utilization coefficient

4.1.2.1.Experiment Approach

This study was conducted at James Cook University (JCU), Marine Aquarium Research Facility. The protein and energy utilization efficiency of tiger grouper was determined by feeding five different ration levels ranging from 0% (starved), 25%, 50%, 75% and 100% of satiated feeding using a commercial marine fish diet. The nutrient profile of the diet is indicated in Table1 and was considered appropriate for tiger grouper of the size used in this study. Grouper initial weight was 336.7 ±3.0 g and the experiment was run for 64 days for the fed group and 14 days for the starved group. Twenty five grouper were each stocked into 300 l cages with 4 cages situated within 3 x 2500 l tanks representing the fed ration levels (25%, 50%, 75%, 100% satiation) and n = 3 cages per ration treatment. The 0 % ration treatment was conducted at the conclusion of the feed trial with n = 3 cages in a 2500 l tank and run for two weeks. Each holding tank was part of a 20,000 l temperature controlled recirculating aquaculture system (RAS). All tanks were exposed to indirect natural light (photoperiod 12L:12D). Temperature (25.6 – 27.8 °C), ammonium (NH4+) (<0.1 mg l-1), dissolved oxygen (>5.0 mg l-1), pH (8.2 – 8.4) and salinity (31 - 35 ppt) were monitored regularly throughout the duration of the experiment.

4.1.2.2.Feeding & Diet Digestibility

Fish were fed 6 mm extruded floating pellets once or twice daily depending on ration size. Any uneaten pellets were counted then netted from cages approximately 45 min after initial feeding. Total daily feed intake was adjusted accordingly.

Following completion of the feed utilization study nutrient and energy apparent digestibility coefficients (ADC's) of the diet were determined. Twenty five 520 g grouper (i.e. representing approximate final weight of satiated group) were each stocked into 300 l cages (n = 4) and fed the re-pelleted diet containing yttrium oxide (Y_2O_3) marker included at 1 g Y_2O_3 / kg diet. Fish were netted from their respective cages and placed into an aerated tank containing a commercial anesthetic AQUI-S ® (dosage approx. 0.01 ml/l) until they lost consciousness. Individual fish were then removed and the feces were collected by stripping technique using gentle pressure along the distal portion of the intestine to expel feces. Care was taken to avoid collecting urine. Faces were collected into a plastic vial at each stripping event and stored frozen at -20 °C. Samples were transferred to a -800 °C freezer prior to freeze drying. Fecal samples were then pooled within replicate cage prior to chemical analysis.

Diet nutrient and energy apparent digestibility coefficients were calculated as:

ADC of dry matter (%) = [1-(Y_2O_3 in diet / Y_2O_3 in feces)] x 100 Eq.2

ADC of nutrients or energy (%) = [1-(Y_2O_3 in diet / Y2O3 in feces x concentration of nutrient or energy in feces / concentration of nutrient or energy in diet)] x 100 Eq.3

4.1.2.3.Sample Preparation & Analyses

Fish were fasted for 48 h prior to sampling for carcass composition. Initial representative samples of 5 fish were collected before the start of the experiment and frozen (-20°C). At the conclusion of the feeding trial all fish were euthanized with an overdose of AQUI-S®, weighed and stored frozen for compositional analyses. Homogenized whole carcass were sub-sampled for dry matter determination. A further sub-sample of homogenate was then freeze dried, finely ground and analyzed for proximate chemical, energy and amino acid profile following standard methods (AOAC, 2016). Compositional changes were estimated by comparing the initial fish carcass samples with those from the feeding trial.

4.1.2.4.Data Analyses

The following performance indices were calculated:

Daily weight gain (g $fish^{-1}$ day^{-1}) = Final body weight (fbw) – ibw / number of days

Daily nutrient gain (g $fish^{-1}$ day^{-1}) = Final carcass nutrient content –initial carcass nutrient content / number of days
Daily energy gain (kJ $fish^{-1}$ day^{-1}) = Final carcass energy content – initial carcass energy content / number of days
Feed Conversion Ratio (FCR) = Total feed intake / Weight gain
Nutrient or energy Retention Efficiencies (RE) = Nutrient or energy gain / Total Digestible Nutrient or Digestible energy intake

Data were expressed as geometric mean body weights (GMBW) and scaled using the metabolic body weight exponent value of 0.7 for protein and amino acid retention data and 0.8 for energy retention data.

The main objective of this study was to better understand dietary protein, energy and amino acid utilization in tiger grouper. The feed ration levels (starved through to satiation feeding) were chosen to elicit a strong dose / response therefore testing for statistical differences among ration treatments was not done. Rather, regression analyses were applied across the data set to determine utilization co-efficients (efficiencies), nutrient cost of growth and maintenance requirements for DP, DE and key amino acids.

4.1.3. Results and Discussion

The feed nutrient and energy composition and corresponding digestibility coefficients are presented in Table 4.1. Digestibility of dietary protein and energy were good at over 80%. All of the dietary essential amino acids were also highly digestible (86%-92%). Dietary taurine had a relatively low digestibility at 65%.

All growth and feed utilization data are presented in Table 4.2. FCR's in the satiated (100%), 75% and 50% restricted rations were excellent ranging from 1.0 to 1.1. Survival at the end of the experiment was 100%.

4.1.3.1.Utilization Coefficients

The daily protein intake to achieve maximum predicted Protein Retention Efficiency (PRE) was 2.0 g DP $kg^{-0.7}$ day^{-1}. A summary of utilization co-efficients, cost of nutrient gain and maintenance requirements are presented in Table 4.3. The utilization efficiency of dietary protein for tiger grouper was 0.58±0.04. The corresponding cost of DP per unit of protein gain was 1.71 g g^{-1} The protein utilization efficiency of tiger grouper was 0.58 and similar to white grouper (Lupatsch and Kissil, 2005), European seabass (Lupatsch, et al., 2001), Asian seabass (Glencross, 2008; Lupatsch and Kissil, 2003), and mulloway (Pirozzi, et al., 2010c). A value of 0.58 equates to a cost of production of 1.71 g DP g^{-1} protein deposition. The DP required to produce a maximum growth response was estimated at was 2.0 g DP $kg^{-0.7}$ day^{-1} which is the same value as that quantified for mulloway at a similar temperature (Pirozzi, et al., 2010c). Growth responses to amino acid intake were generally curvilinear indicating that dietary amino acids were sufficiently supplied in the diet. Essential amino acid requirements for tiger grouper are yet to be quantified; however, requirements for methionine in hybrid grouper (*E. fuscoguttatus*♀ × *E. lanceolatus*♂) ranged from 1.06% based on protein efficiency ratio (PER) to 1.45% based on weight gain (WG) (Li, et al., 2020). The equivalent total sulfur amino acid (TSAA = met + cys) content in the diets by Li et al.(2020) was approximately 1.8 – 2.2 % for PER and WG respectively. The dietary methionine requirement of gold spot grouper is 1.31% based on WG (Luo, et al., 2005b); however, cys data was not presented in that study to estimate TSAA content. The diet used in this study contained 1.17% methionine and a TSAA content of 2.2%.

Table 4.1. Nutrient composition and corresponding Apparent Digestibility Coefficients of the diet (dry matter basis) used for tiger grouper to assess nutrient and energy utilization efficiencies. Composition values expressed as % unless otherwise indicated

Nutrient	Composition (%)	Diet ADCs
Proximates		
Dry Matter	96.95	0.72
Gross Energy (MJ/kg)	21.63	0.82
Protein	52.51	0.84
Fat	13.19	0.96
Ash	7.36	0.23
NFE*	26.93	0.51
Amino Acids		
Alanine	2.94	0.87
Arginine	2.99	0.92
Aspartic acid	4.38	0.82
Cystine	0.79	0.74
Glutamic acid	8.31	0.91
Glycine	2.96	0.87
Histidine	1.51	0.87
Isoleucine	1.52	0.89
Leucine	4.24	0.89
Lysine	3.01	0.88
Methionine	1.17	0.92
Phenylalanine	2.43	0.90
Proline	2.43	0.89
Serine	2.67	0.87
Threonine	2.08	0.86
Tyrosine	1.66	0.86
Valine	2.56	0.87
Taurine	0.21	0.65

*Nitrogen Free Extract calculated by difference; 100 – (protein + fat + ash)

Taurine requirements for grouper are currently unknown. Taurine is recognized as an essential nutrient particularly for marine carnivores fed non-fishmeal diets that are based on soy-protein concentrates (Salze and Davis, 2015; Takagi, et al., 2008). When taurine is deficient in the diet it is associated with green liver syndrome and a decrease in bile pigments and hemolytic biliverdin overproduction. The average dietary taurine requirement of marine species can generally be met at approximately 0.9% inclusion of taurine in the diet (reviewed by Salze and Davis, 2015). While the dietary taurine content in the diets used in this study was only 0.21%, methionine was likely supplied in excess. If methionine can spare the requirement for taurine, as has been demonstrated in other marine species (Candebat, et al., 2020; Ferreira, et al., 2015), then it is unlikely that tiger grouper in this study were deficient in taurine. Clearly the requirements for sulfur amino acids for tiger grouper is an area that requires further investigation, particularly as methionine is often the first limiting amino acid when formulating low fishmeal diets.

Table 4.2. Summary of performance indices of each feed ration group (n=3). Met = Methionine; Lys = Lysine; His = Histidine; Tau = Taurine. RE = Retention efficiency. Feed and nutrient intake expressed on a Dry Matter basis

	Feed Ration					
	0%	**25%**	**50%**	**75%**	**100%**	**Pooled SE**
Performance Indices						
Survival (%)	100	100	100	100	100	
Initial Body Weight (g)	342.41	335.17	335.55	335.42	335.10	0.267
Final Body Weight (g)	330.31	368.27	435.77	484.31	526.92	2.810
Gain (g/fish/day)	-0.81	0.52	1.57	2.33	3.00	0.046
Feed Intake (g/fish/day)	-	0.86	1.73	2.36	3.01	0.028
FCR	-	1.67	1.10	1.01	1.00	0.028
DP Intake (g/fish/day)	-	0.38	0.76	1.04	1.33	0.022
Protein Retention (g/fish/day)	-0.21	0.07	0.32	0.39	0.54	0.023
PRE	-	0.20	0.42	0.37	0.40	0.014
DE Intake (kJ/fish/day)	-	15.37	30.79	42.06	53.64	0.505
Energy Retention (kJ/fish/day)	-12.32	5.63	28.39	35.78	52.67	1.954
ERE	-	0.16	0.43	0.41	0.49	0.050
DMet Intake (mg/fish/day)	-	9.29	18.61	25.42	32.42	0.305
Met Retention (mg/fish/day)	-10.17	0.54	7.75	8.74	12.17	0.823
MetRE	-	0.06	0.42	0.34	0.37	0.06
DLys Intake (mg/fish/day)	-	22.92	45.89	62.69	79.96	0.752
Lys Retention (mg/fish/day)	-24.93	4.39	22.86	25.89	35.72	1.171
LysRE	-	0.19	0.50	0.41	0.45	0.030
DHis Intake (mg/fish/day)	-	11.29	22.61	30.88	39.39	0.371
His Retention (mg/fish/day)	-8.20	1.16	6.12	6.91	9.60	0.314
HisRE	-	0.10	0.27	0.22	0.24	0.017
DTau Intake (mg/fish/day)	-	1.16	2.33	3.18	4.06	0.038
Tau Retention (mg/fish/day)	-2.72	-0.28	0.14	0.52	0.84	0.135
TauRE	-0.24	0.06	0.16	0.21	-0.24	0.066

The dietary energy utilization efficiency (0.63) of tiger grouper is within the range reported for other fish species (0.4 - 0.7; Bureau, et al., 2006). A co-efficient value of 0.63 equates to an energetic cost of production of 1.59 kJ DE kJ^{-1} energy deposition. The DE required to produce a maximum growth response was estimated at 101 kJ DE $kg^{-0.8}$ day^{-1} which is equivalent to mulloway at 107 kJ DE $kg^{-0.8}$ day^{-1} and held at a similar temperature (Pirozzi, et al., 2010c). However temperature has been shown to influence utilization coefficients in yellowtail kingfish (Pirozzi, et al., 2019) which can have a significant impact on predicted requirement (Pirozzi, et al., 2010a).

The size of grouper in this study, ~300 g, was larger than most fish in other published studies yet utilization efficiencies were similar. Body size has been shown not to influence co-efficient values greatly in other marine finfish species (Lupatsch and Kissil, 2003; Lupatsch, et al., 2001; Pirozzi, et al., 2010c) which is an important consideration when using these values to model dietary energy requirements as utilization co-efficient have a significant impact on predicted requirements (Pirozzi, et al., 2010a).

Table 4.3. Selected digestible nutrient and energy utilization coefficient and maintenance values. Data generated from the linear regression of fed treatment groups

Nutrient	Equation	Maintenance Requirement	Cost of Gain	r^2
Energy (kj $kg^{-0.8}$ day^{-1})	0.628*X-15.92	25.34	1.59	0.91
Protein (g $kg^{-0.7}$ day^{-1})	0.584*X -0.371	0.634	1.71	0.95
His (mg $kg^{-0.7}$ day^{-1})	0.297*X - 3.449	11.63	3.37	0.91
Met (mg $kg^{-0.7}$ day^{-1})	0.506*X - 6.873	13.59	1.98	0.90
Lys (mg $kg^{-0.7}$ day^{-1})	0.543*X - 12.54	23.11	1.84	0.91
Tau (mg $kg^{-0.7}$ day^{-1})	0.431*X - 1.651	3.829	2.32	0.79

4.1.3.2.Maintenance Requirements

Maintenance requirements represent the minimum amount of dietary nutrient or energy required to sustain life but not promote growth. Factorial bioenergetic models partition requirements for maintenance and growth to facilitate a comprehensive understanding of overall nutrient requirements. Maintenance energy requirement for tiger grouper was estimated at 25.3 kJ DE $kg^{-0.8}$ day^{-1} which is lower than that (40 – 60 kJ DE $kg^{-0.8}$ day^{-1}; Bureau, et al., 2002). Conversely protein requirements for maintenance for tiger grouper were 0.63 g DP $kg^{-0.7}$ which is higher than other published data of ~0.45 g DP $kg^{-0.7}$ day^{-1} (Glencross, 2008; Lupatsch and Kissil, 2003; Pirozzi, et al., 2010b) but substantially lower than yellowtail kingfish *Seriola lalandi* at 1.7 g DP $kg^{-0.7}$ (Booth, et al., 2010). Apart from inherent species effects such as behaviour and physiology, differences in maintenance energy and protein requirements may be attributed to temperature of culture systems, the quality and digestibility of diets and the type of regression analyses used to calculate the x-intercept. There is scant information on the maintenance requirements for amino acids in fish. However, using a non-linear mixed modelling approach Hua (2013) estimated essential amino acid maintenance requirements for lysine, methionine and histidine at 15.6, 18.4 and 9.8 mg $kg^{-0.75}$ day^{-1} respectively.

4.1.3.3.Carcass Composition

Carcass composition data are presented in Table 4.4. Whole-body protein content remained reasonably conserved at 18.6 % irrespective of the feed level and this is a typical response in fish species (Shearer, 1994). Protein has been shown to vary significantly with ration and also temperature in other species (Pirozzi, et al., 2010c). However, while statistically different the overall difference is quite small at ~1%. Fat content in tiger grouper in the satiated group was 9.7% and higher than that observed in the same species investigated by Shapawi et al. (2014) at 5.5% even though the fat content of the diet used in this study was lower (13% *cf.* 16%) and the protein content similar (52.5% this study *cf.* 50% by Shapawi et al. (2014). This difference is more likely the results of different body sizes in grouper (527 g final body weight this study *cf.* 37 g final body weight best performing diet Shapawi et al. (2014)) as energy composition of fish is known to increase with increasing body size (Pirozzi, et al., 2010a).

Table 4.4. Whole carcass final nutrient and energy composition. (As Is Basis; n=5 fish per cage; n= 3 cages per treatment). Initial carcass composition (fed group). Moisture 66.76 %, Ash 5.20%, Energy 7.62 MJ/kg, Fat 9.30%, Protein 18.45%, Met 0.46%, Tau 0.05%, His 0.31%, Lys 1.30%

	Feed Ration					
	0%	**25%**	**50%**	**75%**	**100%**	**Pooled SE**
Proximates						
Moisture (%)	67.60	68.64	67.21	68.35	67.35	0.37
Protein (%)	19.87	18.09	18.94	17.91	18.26	0.16
Fat (%)	8.64	8.39	9.42	9.39	9.73	0.26
Ash (%)	5.18	4.90	4.43	4.47	4.51	0.42
Energy (MJ kg^{-1})	7.43	7.36	7.80	7.56	8.04	0.19
Amino Acids						
Alanine	1.36	1.24	1.27	1.19	1.22	0.017
Arginine	1.27	1.13	1.16	1.10	1.10	0.013
Cystine	1.80	1.56	1.64	1.54	1.57	0.004
Glutamic acid	0.18	0.15	0.17	0.16	0.16	0.024
Glycine	2.84	2.52	2.63	2.46	2.53	0.029
Histidine	2.02	1.81	1.81	1.69	1.70	0.004
Isoleucine	0.34	0.30	0.33	0.31	0.31	0.008
Leucine	0.63	0.55	0.61	0.56	0.55	0.017
Lysine	1.22	1.13	1.19	1.12	1.14	0.013
Methionine	1.43	1.26	1.34	1.25	1.26	0.007
Phenylalanine	0.51	0.42	0.46	0.43	0.44	0.009
Proline	0.74	0.65	0.69	0.64	0.65	0.013
Serine	1.02	0.99	0.97	0.94	0.93	0.008
Threonine	0.75	0.69	0.72	0.69	0.72	0.006
Tyrosine	0.04	0.04	0.04	0.04	0.04	0.010
Valine	0.80	0.71	0.75	0.71	0.74	0.007
Taurine	0.045	0.038	0.038	0.040	0.040	0.002

4.1.3.4.Development of Growth Model

Laboratory data and published values were collated from approximately 300 individual fish to produce a growth model for tiger grouper ranging from <10 g to approximately 1 kg in body weight. The following equation describes the growth potential of tiger grouper at approximately 28°C:

$$\text{Growth (g fish}^{-1}\text{ day}^{-1}) = 0.08794(BW)^{0.6646} \quad r^2=0.98$$

4.1.3.5.Development of Whole-Body Protein and Energy Composition Model

Whole body protein and energy composition of tiger grouper was established by analyzing whole carcass samples of approximately 200 fish ranging from approximately 20 – 1020 g. Protein composition was reasonably constant irrespective of body size at 17.29 g $100g^{-1}$. Energy composition increased with body weight according to the following equation: Energy composition (kJ g^{-1}) = $5.0364(BW)^{0.0679}$ r^2=0.76

4.1.3.6.Development of a Factorial Model for Tiger Grouper

By applying the above data to Equation 1 the requirements for DP and DE for tiger grouper growing to 1 kg body weight can be determined (Table 4.5).

Growth stanzas were identified using breakpoint regression analyses at approximately 50 and 430 g which indicated a significant shift in nutrient demand and therefore by necessity a change in diet in growing fish (Figure 4.1).

Table 4.5. Digestible protein and energy requirements of tiger grouper grown at 28°C

Live Weight (g)	10	100	200	500	1000
Growth (g fish^{-1} day^{-1})	0.4	1.9	3.0	5.5	8.7
Energy					
MBW (kg$^{-0.8}$)	0.03	0.16	0.28	0.57	1.00
Energy Gain (kJ fish^{-1} day^{-1})	2.4	12.9	21.5	42.0	69.8
DE Maintenance (kJ fish^{-1} day^{-1})	0.6	4.0	7.0	14.6	25.3
DE Growth (kJ fish^{-1} day^{-1})	3.8	20.6	34.2	66.8	111.1
DE Total (kJ fish^{-1} day^{-1})	4.4	24.6	41.2	81.4	136.4
Protein					
MBW (kg$^{-0.7}$)	0.04	0.20	0.32	0.62	1.00
Protein Gain (g fish^{-1} day^{-1})	0.03	0.13	0.21	0.39	0.63
DP Maintenance (g fish^{-1} day^{-1})	0.07	0.32	0.51	0.95	1.50
DP Growth (g fish^{-1} day^{-1})	0.12	0.56	0.88	1.62	2.57
DP Total (g fish^{-1} day^{-1})	0.15	0.68	1.09	2.01	3.20
DP:DE (g DP MJ DE^{-1})	32.7	27.8	26.4	24.7	23.5

The theoretical requirement for the ratio of DP to DE for growing tiger grouper up to 1 kg body weight can be expressed as:

Tiger grouper DP:DE requirement (g DP MJ DE^{-1}) = $38.75(BW)^{-0.073}$

Figure 4.1. Theoretical requirement for the ratio of DP to DE for growing tiger grouper up to 1 kg body weight at 28°C. Breakpoint regression analysis (linear-linear-linear) identified key growth stanzas at <50.8 g, 50.8-432.4 g and >432.4 g

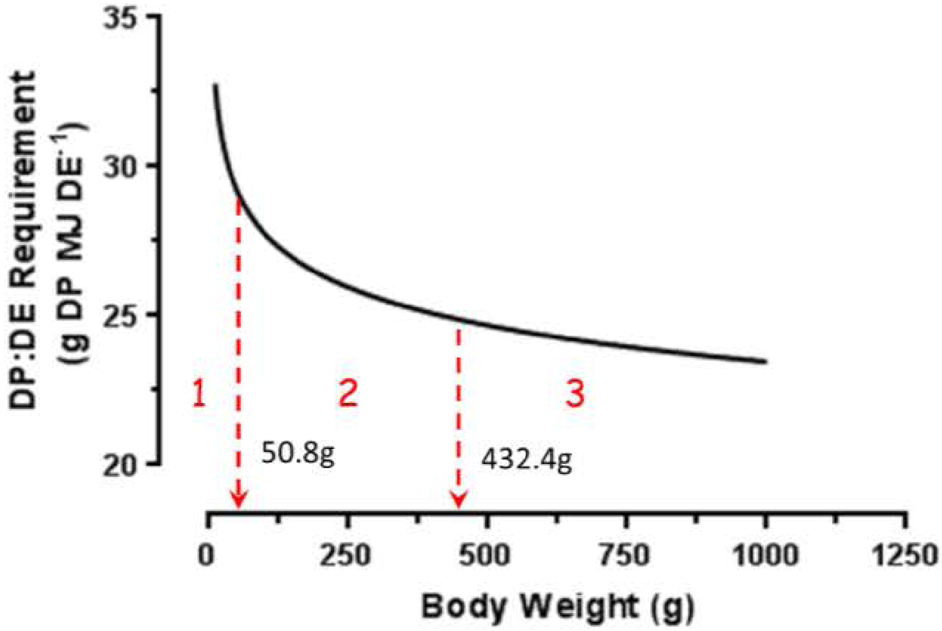


Figure 4.1 indicates the constant change in nutrient requirement for growing fish. In practical terms however it is not possible to meet the changing nutritional requirement of grouper with multiple commercial diets throughout the entirety of grow-out production. A compromise must be considered which balances feed production logistics and price with the nutritional demands of the species. Table 5 provides suggestions for practical diet specifications for DP and DE for tiger grouper growing to 1 kg. Suggested diet specifications for; fingerlings to 100 g = 51.2% DP and 16.0 MJ kg^{-1} DE; 100-500g = 44.8% DP and 16.0 MJ kg^{-1} DE; 500-1000g = 43.2% DP and 18.0 MJ kg^{-1} DE.

4.1.4. Conclusion

Modeling the data collated from the above studies provides information describing the requirement for DP and DE for tiger grouper growing to 1 kg in body weight. This will aid in feed formulation decisions targeting appropriate nutrient specifications at different growth stanzas for this species. Predictions on feed intake and FCR's (Table 4.6) will facilitate better on farm feed management for tiger grouper by providing baseline data of expected intake and feed conversion efficiencies.

The application of nutrient requirement and feed demand models on-farm should be conducted with due diligence as many variables can influence nutrient demand and therefore the accuracy of bioenergetic models; this includes fish size, ingredient type, temperature and other abiotic factors etc. Therefore, extrapolating beyond the parameter range of the modeled data set should be done with caution. In this study fish were sampled up to 1kg and growth studies were conducted at 28°C. The presented growth model is based on a limited dataset. A more robust factorial model would therefore consider larger fish of several kg and growth at a number of different temperatures and is a suggested area for future research.

Table 4.6. Diet specification for digestible protein (DP) and digestible energy (DE) content for tiger grouper aquafeeds at three digestible energy densities; 17,18 and 19 MJ DE kg^{-1} and three digestible protein levels; 510, 468 and 437 g kg^{-1} Specifications iteratively derived using a bioenergetic factorial modelling approach. Practical diet assignment at three key growth stanzas and corresponding diets indicated within each shaded box. <100g (Diet 1); 100-500g (Diet 2); 500-1000g (Diet 3)

	Diet 1		Diet2		Diet 3	
Live weight (g)	10	50	100	200	500	1000
DE content (MJ/kg)	17.0	17.0	17.0	17.0	17.0	17.0
DP content (g/kg)	510.0	510.0	442.0	442.0	391.0	391.0
Intake (g/fish/day)	0.26	0.86	1.45	2.42	4.79	8.02
Intake (%BW/day)	2.61	1.73	1.45	1.21	0.96	0.80
Expected FCR	0.64	0.73	0.77	0.81	0.88	0.93
DE content (MJ/kg)	18.0	18.0	18.0	18.0	18.0	18.0
DP content (g/kg)	540.0	540.0	468.0	468.0	414.0	414.0
Intake (g/fish/day)	0.25	0.82	1.37	2.29	4.52	7.58
Intake (%BW/day)	2.47	1.63	1.37	1.14	0.90	0.76
Expected FCR	0.61	0.69	0.73	0.77	0.83	0.87
DE content (MJ/kg)	19.0	19.0	19.0	19.0	19.0	19.0
DP content (g/kg)	570.0	570.0	494.0	494.0	437.0	437.0
Intake (g/fish/day)	0.23	0.77	1.29	2.17	4.28	7.18
Intake (%BW/day)	2.34	1.55	1.29	1.08	0.86	0.72
Expected FCR	0.58	0.65	0.69	0.73	0.78	0.83

4.2. Apparent Digestibility Coefficients of SBM and SPC for Tiger Grouper

4.2.1. Introduction

There are very few published data documenting the digestibility of soy ingredients in diets for grouper. Of the published data that are available most have focused on gold spot grouper (Eusebio, et al., 2004; Lin, et al., 2004; Zhuo, et al., 2016). Some data are available on alternative protein source digestibility in tiger grouper (e.g.

Usman, et al., 2007) but none have specifically considered SBM or SPC or amino acid digestibility for this species. The objective of this study was to determine the digestibility of proximate composition (protein, fat, energy and dry matter) and essential amino acids of SBM and SPC diets when fed to tiger grouper.

4.2.2. Material and Methods

This study was conducted at JCU Marine Aquarium Research Facility. The approach to determining soy diet and ingredient digestibility used the substitution method replacing either 10% or 30% dry matter basis of a reference diet with solvent extracted SBM (i.e. SBM10 or SBM30) or SPC (i.e. SPC10 or SPC30) to create four soy test diets (Table 4.7). The basal diet was a commercial marine fish diet ground and repelleted (screw-pressed; Dolly, La Monferrina) to contain 0.1% yttrium oxide (Y_2O_3) marker. The nutrient composition of the diets and soy ingredients are presented in Table 8.

Analyses of the essential amino acid profiles demonstrated that, with the exception of methionine, SPC contained a greater amount of all essential amino acids when compared to the commercial marine fish basal diet.

Table 4.7. Ingredient composition (g/kg) of diets

	Diet				
Ingredient	**Basal Diet**	**SBM10**	**SBM30**	**SPC10**	**SPC30**
Basal Diet	999	899.1	699.3	899.1	699.3
SBM	0	100	300	0	0
SPC	0	0	0	100	300
Yttrium Oxide	1	0.9	0.7	0.9	0.7
Sum	1000	1000	1000	1000	1000

Fifteen tiger grouper each weighing 774.8±76.1 g were stocked into 300L cages (n = 3 cages per diet treatment) and acclimated on the test diets for one week before stripping was undertaken to collect fecal material. Diets and fecal material were analyzed for proximate and amino acid composition and the difference in the ratio of the marker to the nutrient in the feed and feces indicated the apparent digestibility of the nutrient of interest.

Diet nutrient and energy apparent digestibility coefficients (ADC's) were calculated using Eq.2 and Eq.3 above.

In addition, ingredient digestibility was calculated as:

$$ADC_{ING}\ (\%) = [(Nutr_{TD} * AD_{TD}) - (PRD * Nutr_{RD} * AD_{RD})] / [(P_{ING} * Nutr_{ING})] \quad \text{Eq.4}$$

where ADC_{ING} = apparent digestibility of nutrient or gross energy in the test ingredient; $Nutr_{TD}$ = the nutrient or gross energy concentration in test diet; AD_{TD} = the apparent digestibility of the nutrient or gross energy in the test diet; PRD = proportional amount of reference diet; $Nutr_{RD}$ = the nutrient or gross energy concentration in the reference diet; AD_{RD} is the apparent digestibility of nutrient or gross energy in the reference diet; P_{ING} = proportional amount of test ingredient; $Nutr_{ING}$ is the nutrient or gross energy concentration in the test ingredient (Sugiura, et al., 1998).

4.2.3. Results and Discussion

Apparent digestibility coefficients are presented for diets (Table 4.9) and ingredients (Table 4.10). Overall SBM and SPC diet digestibility for tiger grouper were generally good, although the higher 30% inclusion diets tended to have a lower digestibility value than the 10% inclusion diets (Table 9). SPC protein and amino acid ADC's tended to be greater than SBM values. SBM amino acid diet digestibility values compare favorably to those established for gold spot grouper (Lin, et al., 2004), Asian sea bass (Booth and Allan, 2010) and spotted rose snapper (Hernández, et al., 2015). There are few studies considering SPC digestibility in aquaculture species; however, SPC protein and amino acid diet digestibility in tiger grouper were found to be similar to some salmonid species (Chowdhury, et al., 2012). The improved digestibility coefficients of SPC compared to SBM are likely an artefact of the ingredient refining process and a reduction of antinutritional factors and polysaccharides present in the less refined SBM product.

The ingredient proximate and amino acid ADC's were generally good displaying a similar trend to that of diet ADC'S, i.e. SPC tending to be more digestible than SBM (Table 4.10). This was also the case for most of the essential amino acids. Similar responses for SBM and SPC digestibility has been demonstrated for Asian sea bass (Booth and Allan, 2010) which also indicated a good capacity to digest soy proteins. Both tiger grouper and Asian sea bass are tropical eurythermal and euryhaline species (Cheng, et al., 2013; Rimmer and Russell, 1998) occupying a similar trophic ecology; therefore, in the absence of species specific empirical data, a reasonable starting point for feed formulation would be to consider ADC values generated from species occupying similar trophic levels. This contrasts with yellowtail kingfish, a pelagic marine carnivore, where ADC values for protein and energy for SPC (Dam, et al., 2019) and SBM (Bowyer, et al., 2013a) are comparatively lower. There were some anomalies with ingredient ADC values such as those >100% (Table 4.10). This can sometimes occur when examining ADC's at the ingredient level and can be caused by a number of reasons including ingredient interactions, mixing and/or analytical errors which are compounded through the application of ingredient level digestibility calculations (Booth and Pirozzi, 2018; Glencross, et al., 2007); interestingly these anomalous values occurred predominantly with the low 10% soy inclusion which tends to support this hypothesis.

4.2.4. Conclusion

This study determined the proximate and amino acid digestibility of SPC and SBM for tiger grouper at two different inclusion levels. These data will assist in the formulation of Diets on a digestible basis to achieve targeted nutrient specifications. SPC was shown to be more digestible than SBM and, generally, low dietary inclusion levels tended to improve ADC values, although this was variable depending on the nutrient of interest and tended to be more pronounced for SBM. When using these ingredients in aquafeeds formulators should consider which nutrients are preferentially important to them for the species of interest, for e.g. targeting specific amino acid contribution over total protein. While soy proteins have been utilized extensively in aquafeeds for many years there is still a surprising lack of information on ingredient digestibility, particularly at different inclusion levels. This is a significant oversight for the industry as clearly there are potential interactions which can affect assumed digestibility of an ingredient in the diet.

4.3. Optimal Inclusion Level of SBM and SPC for Gold Spot Grouper

4.3.1. *Introduction*

The use of soy protein as a partial fishmeal replacement has been extensively studied for many aquaculture species and the limiting factors for upper tolerance thresholds are well known (Francis, et al., 2001). Surprisingly there are limited published data available assessing the inclusion of SBM in diets for grouper and even less when considering SPC. Of those studies most have shown a negative correlation with increasing soy inclusion and performance when substituting for fishmeal (FM) as the main protein source; however, almost without exception these studies have substituted FM with soy products without maintaining nutritionally balanced diet specifications with regard to essential nutrients, particularly when considering methionine (e.g. Chen, et al., 2019; Faudzi, et al., 2017; Shapawi, et al., 2013; Wang, et al., 2017). Without maintaining a nutritionally balanced diet it is problematic to decide with certainty what the upper threshold or optimal inclusion of a soy ingredient might be.

Brewer's yeast is a protein commonly used in small quantities particularly by SE Asian feed manufacturing companies. It is a cheap and useful protein source to partially replace fishmeal (e.g. Oliva-Teles and Goncalves, 2001; Pongpet, et al., 2016) and may also have beneficial prebiotic qualities as it contains β-glucans and polysaccharides (e.g. Burr, et al., 2008). The aim of this study was to evaluate the substitution of FM with SBM or SPC at 0, 10, 20, 30 or 40% inclusion and to also investigate a 20% brewer's yeast diet on various performance indices of gold spot grouper including growth, feed conversion efficiencies, nutrient retention, digestibility and digestive histology.

4.3.2. *Materials and Methods*

4.3.2.1.Experiment Design

1.1.1.1 This study was conducted at NSW Department of Primary Industries, Ports Stephens Fisheries Institute (PSFI). Diets were formulated to be nutritionally balanced with respect to methionine and taurine and to supply adequate crude protein, fat and dietary energy for gold spot grouper based on current available requirement data (Luo, et al., 2005a; Luo, et al., 2005b; National Research Council, 2011). Four SBM diets at (SBM10-40), four SPC diets (SPC10-40), a brewer's yeast 20% inclusion diet (BY20) and a fishmeal Control diet (0% soy protein). Diet specifications for all diets contained on average: 50.9% crude protein, 11.8% crude fat, 20.5 MJ/kg gross energy, 3.3% lysine, 1.4% methionine, 1.0% taurine. Diet formulations are presented in Table 4.11.

1.1.1.2 Raw ingredients were selected as those typically used in commercial grouper feed production in South East Asia and the proximate and amino acid composition of the main protein sources are presented in Table 4.12.

At the time of conducting the feed trials in this report USA SBM and SPC raw ingredients were unavailable in Australia. SBM was sourced through an Australian agrifeed company and was of Argentinian origin. SPC was sourced from ADM Australia and was of EU origin.

Three replicate 200 l cages per dietary treatment were stocked with 44 individual grouper (mean initial weight = 84.0±0.9 g). Cages were held within two 10 kl tanks with one dietary treatment compliment per tank. Tanks were maintained on a common saltwater recirculation system. Due to unforeseen technical problems growth and feed intake data was potentially compromised in one of the 10 kl tanks, therefore analyses was conducted on n=2 replicate cages. The culture temperature was (mean±stdev) 27.7±0.8 ºC; $NH3/4^+$ 0.3±0.1; pH 7.9±0.1; salinity 33.4±0.2; DO 7.2±1.3 mg l^{-1}. An initial sample of 10 fish were also taken for proximate composition and gut histology. Experiment fish were fed daily to satiation for eight weeks before being subsampled (n=15 fish) for carcass composition and histology.

Table 4.8. Nutrient composition (dry matter basis) of the basal diet and soy ingredients used in the tiger grouper digestibility trial. The basal diet was a reground commercial marine fish feed repelleted to include 0.1% yttrium marker. Diet and SBM (Argentinian origin) supplied by Ridley Corporation Ltd. Narangba QLD 4504 Australia. SPC Soycomil-k. ADM Animal Nutrition Pty Ltd North Ryde NSW 2113 Australia

	Ingredient		**Diet**				
Nutrient	**SBM**	**SPC**	**Basal Diet**	**SBM10**	**SBM30**	**SPC10**	**SPC30**
Dry Matter	88.81	90.55	96.87	97.57	98.23	98.29	97.66
Crude protein	58.87	79.56	64.36	58.86	57.07	58.64	62.96
Crude Fat	3.69	2.00	10.71	9.94	7.97	9.28	7.26
Gross energy (MJ/kg)	19.84	20.15	22.93	20.98	20.41	20.82	20.74
Ash	8.34	7.36	10.88	9.86	9.30	9.64	9.06
Alanine	2.59	3.43	3.81	3.49	3.17	3.52	3.44
Arginine	4.34	5.94	3.11	3.21	3.32	3.32	3.76
Aspartic acid	6.91	9.21	5.31	5.30	5.42	5.47	6.16
Cystine	0.84	1.03	0.75	0.69	0.66	0.68	0.74
Glutamic acid	11.36	15.24	10.31	9.56	9.54	9.79	10.75
Glycine	2.48	3.28	3.03	2.87	2.70	2.92	2.96
Histidine	1.54	2.04	2.01	1.93	1.79	1.96	1.94
Isoleucine	2.47	3.36	1.80	1.96	2.00	1.99	2.24
Leucine	4.39	5.99	5.53	4.99	4.72	5.11	5.22
Lysine	3.61	4.95	4.12	3.94	3.76	4.05	4.17
Methionine	0.80	1.06	1.58	1.39	1.22	1.39	1.31
Phenylalanine	2.86	3.95	3.02	2.86	2.76	2.89	3.07
Proline	3.39	4.67	3.03	3.45	3.44	3.45	3.70
Serine	3.27	4.40	2.88	2.76	2.75	2.85	3.12
Threonine	2.41	3.27	2.55	2.48	2.36	2.52	2.65
Tyrosine	2.09	2.64	2.04	1.81	1.75	1.84	2.05
Valine	2.54	3.46	3.02	2.97	2.80	3.01	3.03

4.3.2.2.Diet Manufacture

All experiment diets were made using laboratory scale equipment. Prior to making the experiment diets, each ingredient was milled using a high-speed hammer mill, fitted with a 1.6 mm screen (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, and Australia). Wheat flour and starches was autoclaved at 121°C for 2 min. Supplements and processed raw materials were then dry mixed in a Hobart mixer (Hobart Mixer; Troy Pty Ltd, Ohio, USA) before the addition of oil and fresh water, forming a moist dough. The dough was then screw-pressed into 6mm sinking pellets (Dolly, La Monferrina, Castell'Alfero, Italy) and oven dried at ~60°C to achieve a final moisture content <10%. This feed making process was also applied to feed trial section 0 below.

Table 4.9. Diet digestibility coefficients (%) of proximate and essential amino acids of soybean meal and soy protein concentrate diets at 10% or 30% inclusion compared to a commercial marine fish diet when fed to tiger grouper. Treatments within rows sharing superscript letters are not significantly different; p>0.05

Nutrient	Basal Diet	SBM10	SBM30	SPC10	SPC30
Dry Matter	70.6±1.2	74.9±1.4	66.4±3.0	74.8±1.5	74.1±1.5
Crude protein	83.3±0.7^{ab}	83.9±0.9^{ab}	80.6±1.4^{a}	84.6±0.6^{ab}	86.5±1.0^{b}
Crude Fat	94.2±0.2^{b}	95.2±0.2^{b}	90.9±0.8^{a}	95.4±0.6^{b}	94.3±0.5^{b}
Gross energy	82.5±0.4^{ab}	84.3±1.0^{b}	78.5±1.8^{a}	85.1±0.8^{b}	84.8±0.8^{b}
Alanine	86.91±0.46^{b}	84.72±1.09^{ab}	80.61±1.27^{a}	86.07±0.58^{b}	86.22±1.16^{b}
Arginine	90.7±0.4^{a}	90.0±0.7^{a}	88.7±1.0^{a}	91.6±0.3^{ab}	93.6±0.5^{b}
Aspartic acid	83.20±0.57^{ab}	83.66±1.05^{ab}	81.01±1.49^{a}	85.14±0.65^{ab}	87.22±0.99^{b}
Cysteine	78.09±1.22^{ab}	81.72±1.24^{b}	73.92±2.23^{a}	81.45±1.28^{b}	84.15±1.17^{b}
Glutamic acid	91.39±0.22^{bc}	90.58±0.38^{ab}	88.79±0.73^{a}	91.57±0.27^{bc}	92.63±0.54^{c}
Glycine	85.86±0.53^{ab}	84.36±1.02^{ab}	81.06±1.44^{a}	86.40±0.55^{b}	87.19±0.94^{b}
Histidine	84.6±0.6^{bc}	80.4±1.4^{ab}	75.6±1.6^{a}	84.5±0.4^{bc}	85.6±1.1^{c}
Isoleucine	89.2±0.5^{ab}	89.4±0.5^{ab}	87.3±1.1^{a}	90.5±0.5^{ab}	92.0±0.5^{b}
Leucine	88.4±0.5^{b}	85.1±1.1^{ab}	81.6±1.5^{a}	87.5±0.3^{b}	88.4±1.0^{b}
Lysine	87.7±0.5^{b}	85.6±1.0^{ab}	82.6±1.3^{a}	87.6±0.3^{b}	88.9±1.0^{b}
Methionine	92.1±0.2^{b}	91.2±0.5^{b}	88.9±0.8^{a}	92.7±0.3^{b}	93.1±0.4^{b}
Phenylalanine	88.5±0.3^{bc}	84.8±1.2^{ab}	82.5±1.1^{a}	87.4±0.3^{bc}	89.2±1.0^{c}
Proline	91.13±0.75	91.24±0.61	90.47±0.73	91.30±0.50	92.90±0.49
Serine	87.49±0.39^{bc}	86.34±0.98^{ab}	83.66±1.13^{a}	87.95±0.48^{bc}	89.66±0.89^{c}
Threonine	86.3±0.5^{b}	84.8±1.0^{ab}	81.1±1.3^{a}	86.5±0.6^{b}	87.7±0.9^{b}
Tyrosine	85.76±0.25^{ab}	87.48±0.49^{ab}	84.61±1.13^{a}	89.04±0.59^{bc}	91.00±0.74^{c}
Valine	85.7±0.6^{b}	82.7±1.3^{ab}	79.1±1.4^{a}	85.5±0.5^{b}	86.3±1.2^{b}

4.3.2.3.Digestibility

At the conclusion of the growth phase of the study the remaining fish were then used for determination of diet digestibility using the same methods applied in Section 4.1.2.2.

4.3.2.4.Performance Indices

Calculations of fish weight and body composition were based on wet values and feed data were adjusted for dry matter.

Feed intake (g/fish) = (Total feed given-Total uneaten feed)/(Fish number)

Weight gain (%) = Final fish weight-Initial fish weight/ Initial fish weight x 100

Feed Conversion Ratio = Weight of consumed food/weight gain

Protein Retention Efficiency (PRW) = Whole body protein gain/ digestible protein consumed

Specific growth rate (% per day) = [(ln final weight-ln initial weight)/days] x 100

Relative Gut Length (RGL) = gut length (mm)/ Body Length x 100)

Hepatosomatic Index (HSI) = liver weight (mm)/ Body Weight x 100)

Table 4.10. Ingredient digestibility coefficients (%) of proximate and essential amino acids of soybean meal and soy protein concentrate ingredients at 10% or 30% inclusion. Treatments within rows sharing superscript letters are not significantly different; p>0.05

Nutrient	SBM10	SBM30	SPC10	SPC30
Dry Matter	117.4 ± 14.7^{b}	$55.8 \pm 10.6a$	114.5 ± 15.4^{b}	82.9 ± 5.4^{ab}
Crude protein	90.8 ± 9.7	73.7 ± 5.2	94.7 ± 4.9	92.5 ± 2.8
Crude Fat	122.4 ± 4.8^{b}	68.6 ± 6.2^{a}	156.3 ± 28.7^{b}	96.0 ± 7.3^{b}
Gross energy	103.4 ± 11.7^{b}	67.7 ± 6.6^{a}	111.6 ± 8.6^{b}	91.2 ± 3.0^{ab}
Alanine	55.10 ± 15.73	58.57 ± 5.73	77.64 ± 6.37	84.43 ± 4.18
Arginine	85.0 ± 5.1^{a}	85.2 ± 2.8^{a}	95.5 ± 1.6^{ab}	97.1 ± 1.1^{b}
Aspartic acid	86.97 ± 8.49^{a}	77.02 ± 4.21^{a}	95.21 ± 4.03^{a}	92.63 ± 2.33^{a}
Cysteine	111.37 ± 11.37^{b}	65.08 ± 6.95^{a}	103.64 ± 9.70^{b}	94.51 ± 3.18^{ab}
Glutamic acid	83.82 ± 3.55^{a}	83.18 ± 2.31^{a}	92.71 ± 1.93^{ab}	94.58 ± 1.40^{b}
Glycine	67.53 ± 12.48	67.06 ± 5.65	90.86 ± 5.13	90.04 ± 2.98
Histidine	30.0 ± 18.5^{a}	47.8 ± 6.6^{ab}	83.82 ± 3.84^{b}	88.1 ± 3.7^{b}
Isoleucine	90.8 ± 3.7^{ab}	84.0 ± 3.0^{a}	96.7 ± 3.1^{b}	95.4 ± 1.1^{ab}
Leucine	46.2 ± 14.3^{a}	61.2 ± 6.0^{ab}	79.8 ± 2.4^{ab}	88.3 ± 3.1^{b}
Lysine	63.2 ± 11.7^{a}	68.8 ± 4.9^{ab}	87.0 ± 2.5^{ab}	91.0 ± 2.9^{b}
Methionine	74.7 ± 9.6^{ab}	73.6 ± 4.6^{a}	101.0 ± 4.5^{c}	96.5 ± 1.9^{bc}
Phenylalanine	49.2 ± 13.1^{a}	67.5 ± 3.9^{ab}	80.1 ± 2.0^{ab}	90.6 ± 2.7^{b}
Proline	92.12 ± 5.66	89.05 ± 2.29	92.29 ± 3.42	95.59 ± 1.24
Serine	77.01 ± 8.40	75.60 ± 3.51	90.68 ± 3.35	92.98 ± 2.25
Threonine	69.3 ± 10.7	68.0 ± 4.7	87.7 ± 4.6	90.2 ± 2.6
Tyrosine	102.83 ± 4.92^{b}	81.96 ± 3.76^{a}	111.85 ± 4.68^{b}	100.45 ± 2.07^{b}
Valine	50.3 ± 15.3^{a}	60.6 ± 5.5^{ab}	83.7 ± 4.4^{ab}	87.6 ± 3.6^{b}

4.3.2.5.Data Analyses

Data was statistically compared using NCSS-8.0.23 after assumptions on the normality and homogeneity of data were met. ANOVA and Tukey-Kramer post-hoc multiple comparison tests were considered significant at $p<0.05$. Regression analyses was applied to determine the relationship between soy inclusion level and performance indices.

4.3.2.6.Histological Analysis

At the conclusion of the growth trial key digestive tissue; liver, pylorus caeca and hindgut were sampled from n=3 fish per cage + initial sample (n=3), preserved in a buffer solution of 10% formalin. Preserved tissue samples were then H&E stained, sectioned and slide mounted. Histology images were digitized for examination using the CaseViewer version 2.4 for Windows (3DHISTECH). Histological assessment was conducted by Dr Zoe Spiers, Veterinary Pathologist, Elizabeth Macarthur Agricultural Institute, Menangle NSW 2568. Australia.

Liver: The width of each hepatocyte was measured from the left cell wall to the right cell wall at the height of the nucleus on the horizonal plane of the scanned slide. The heights of 10 randomly selected hepatocytes were measured per fish. The average height was calculated. A comment was noted if there were any unusual or pathological features in the sections examined.

Intestine: The height of each villus was measured from the top of the villus to the top of the lowest enterocyte adjacent to the villi. The heights of 20 villi were measured per fish. The average height was calculated. A comment was noted if there were any unusual or pathological features in the sections examined.

Pyloric caeca: The sections of pyloric caeca were examined and severity of leukocytic infiltrates (minimum thickness of leukocytes in the mucosa and maximum thickness of leukocytes in the mucosa), the prevalence of goblet cells (subjectively measured as low, medium or high), and comments for any unusual findings were noted. The following reference photomicrographs (Figure 4.2, Figure 4.3, and Figure 4.4) were used for grading of goblet cell prevalence.

4.3.3. Results and Discussion

There was 100% survival at the conclusion of the eight week feed trial. Performance indices are summarized in Table 4.13. Growth and feed conversion efficiencies were excellent across all diet treatments. On average all diet treatments except for SBM40 and SPC40 approximately tripled in body weight. Fish fed SBM30 were significantly larger than those fed the SBM40 and SPC40 diets. Fish fed the BY20 diet were the best performing and significantly larger than fish fed any of the other diets with the exception of SBM30. There were no differences among grouper fed SBM and SPC diets with up to 40% inclusion when compared to the FM control diet. Breakpoint analysis indicated an inclusion of approximately 30.8% for SBM and 28.9% for SPC as optimal for growth under laboratory conditions in this study (Figure 4.5). There was a strong negative correlation with hepatosomatic index and increasing soy inclusion (Table 4.13, Figure 4.7).

While grouper are considered a carnivorous species they have a relative gut length far longer than that of pelagic marine carnivores such as yellowtail kingfish (*Seriola lalandi*) (Figure 4.6). This may be indicative of a greater omnivorous capacity than that of a strict carnivore (e.g. Wagner, et al., 2009); an elongated intestinal tract may promote greater nutrient absorption through a prolonged gut transit time.

Table 4.11. Diet formulations, dry matter basis

Ingredients	Control	SBM10	SBM20	SBM30	SBM40	SPC10	SPC20	SPC30	SPC40	BY20
Fishmeal	48	38	28	18	8	38	28	18	8	18
SBM	0	10	20	30	40	0	0	0	0	30
SPC	0	0	0	0	0	10	20	30	40	0
Blood meal	6.3	5.2	5.9	6.7	7.5	5.1	5.4	6.9	8	6.26
Brewer's Yeast	4	4	4	4	4	4	4	4	4	20
Choline Chloride (70%)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Corn Gluten	1.7	5	5.1	5.5	6	2.7	1	0.6	0	1.07
Diatomaceous Earth	0.6	2.2	2.5	2.8	3.1	1.9	3	4.7	6.2	2.24
Fish Oil	2.6	3.4	4.2	4.6	4.7	3.5	4.5	5.8	7	4.56
Krill Meal	5	5	5	5	5	5	5	5	5	5
Maize Starch	9.5	7.8	6.5	5.5	4.6	10	9.8	9.6	9.5	3.05
Methionine	0.2	0.4	0.5	0.5	0.5	0.2	0.3	0.4	0.5	0.47
NaH2PO4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Poultry meal	8	8	8	8	8	8	8	5	3	3
Rovimix Stay-C	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Soy Lecithin	2	2	2	2	2	2	2	2	2	2
Taurine	0.8	0.9	1	1	1	0.9	0.9	1	1	0.94
Vitamin premix^	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Wheat Flour	9.6	6.4	5.6	4.7	3.9	7	6.4	5.3	4.1	1.71

^ Ridley Corporation Ltd; marine fish vitamin & mineral premix, formulation undisclosed

Table 4.12. Raw ingredient nutrient composition of the main protein sources. Dry matter basis

	Blood Meal[a,1]	Brewer's Yeast[b]	Corn Gluten[a]	Fishmeal[a,2]	Krill Meal[a]	Poultry meal[a]	SBM[a,3]	SPC[c]
Dry Matter	92.0	97.0	90.6	93.8	93.4	92.9	87.6	91.6
Ash	1.8	5.6	2.9	23.5	12.3	13.7	6.8	6.2
GE (MJ/KG)	24.3	19.9	23.7	18.8	23.6	22.1	19.5	20.3
Protein	97.8	47.9	71.4	65.3	61.6	67.9	54.0	72.5
Fat	0.5	2.8	3.5	9.7	20.2	12.4	2.6	1.2
Alanine	7.1	2.8	5.3	3.9	3.2	4.0	2.3	3.1
Arginine	4.0	2.4	1.9	4.5	3.7	4.6	3.9	5.3
Aspartic acid	9.1	4.7	3.9	5.6	6.4	5.1	6.3	8.4
Cysteine	0.0	0.5	0.9	0.5	0.5	0.1	0.8	0.9
Glutamic acid	8.1	7.5	13.8	7.6	8.0	8.3	10.3	13.7
Glycine	3.9	2.1	1.7	4.2	2.8	5.9	2.2	3.0
Histidine	5.6	1.0	1.2	2.5	1.2	1.5	1.4	1.8
Isoleucine	0.8	2.0	2.3	2.8	2.9	2.7	2.1	3.0
Leucine	11.6	3.1	10.2	2.5	4.7	4.8	3.9	5.5
Lysine	8.7	3.5	1.0	2.1	4.2	3.9	3.2	4.5
Methionine	1.5	0.7	1.3	1.5	1.8	1.2	0.8	1.0
Phenylalanine	6.7	2.0	4.0	2.1	3.0	2.7	2.7	3.6
Proline	3.6	2.2	8.8	3.8	3.2	4.4	3.1	4.8
Serine	5.1	2.4	3.4	2.5	2.7	3.5	3.0	4.0
Taurine	0.0	0.0	0.0	0.2	0.5	0.3	0.0	0.0
Threonine	4.9	2.3	2.1	2.7	2.9	2.7	2.1	2.9
Tyrosine	2.9	1.6	3.0	4.7	2.7	1.9	2.0	2.4
Valine	8.7	2.3	2.6	3.9	2.9	3.7	2.2	3.0

[a] Ingredients supplied by Ridley Corporation Ltd. Narangba QLD 4504 Australia.
[1] Blood meal ring dried
[2] Solvent extracted; Argentinian origin
[3] Reclaimed tuna trimmings
[b] Supplied by Farmers Warehouse, Singleton NSW 2330 Australia.
[c] Soycomil-K. ADM Animal Nutrition Pty Ltd North Ryde NSW 2113 Australia. EU origin

Figure 4.2. Low goblet cell prevalence in pyloric caeca section

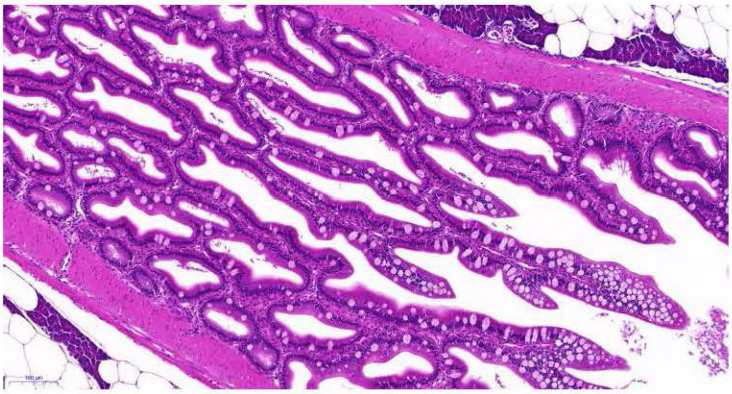


Figure 4.3. Medium goblet cell prevalence in pyloric caeca section

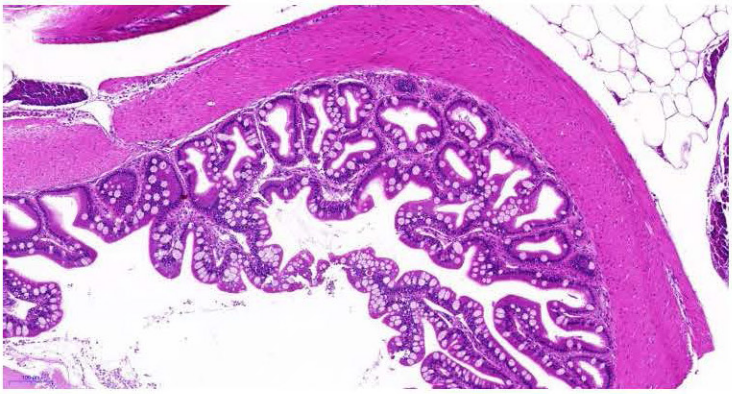


Figure 4.4. High goblet cell prevalence in pyloric caeca section

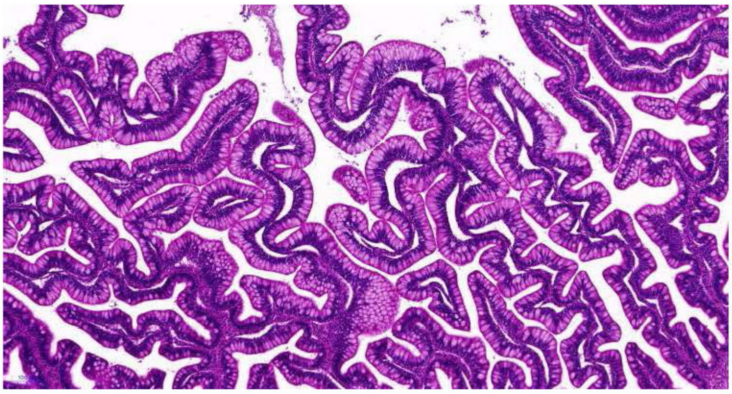


Figure 4.5. Growth response of gold spot grouper when fed increasing levels of SBM or SPC compared to a fishmeal control diet after eight weeks. No statistical difference was found when comparing growth of gold spot grouper fed any of the soy diets to those fed the fishmeal control diet (Ctrl). Fish fed the 20% brewer's yeast 30% SBM diet (BY) were significantly larger than the Ctrl. Data shown as average (n=2) ±SE. Breakpoints at 30.8% (r^2=0.76) and 28.9% (r^2=0.68) for SBM and SPC respectively

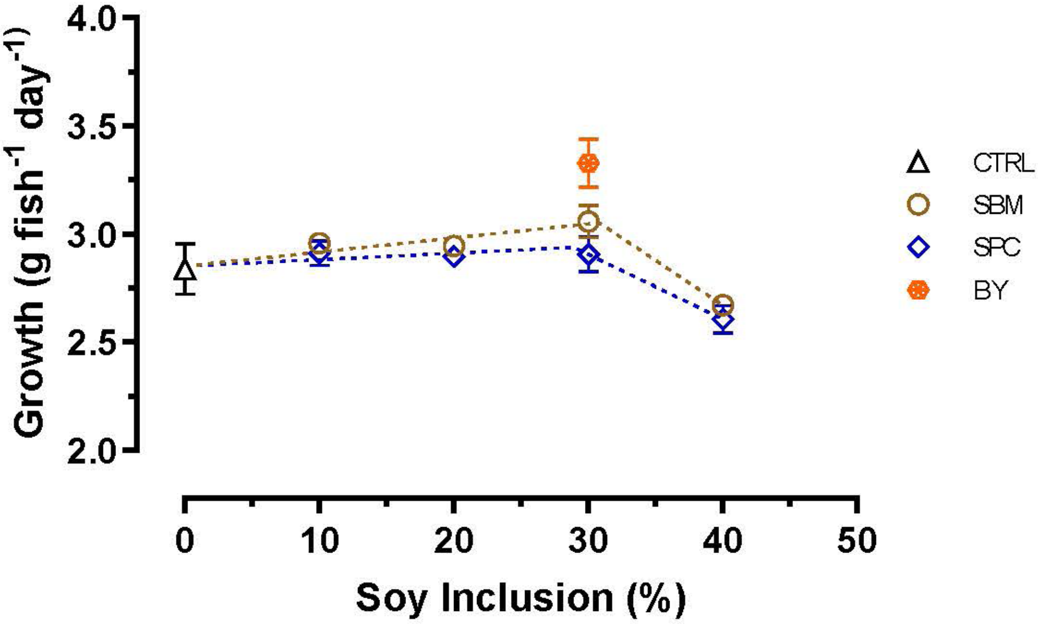


Figure 4.6. Comparative gut length of yellowtail kingfish *Seriola lalandi* (136 mm; top) and gold spot grouper (516 mm; bottom) of fish with similar body weight (307 and 304 g respectively) and total length (305 and 285 mm respectively)

Table 4.13. Summary of performance of gold spot grouper fed varying levels of SBM or SPC diets. SGR = specific growth rate; FCR = feed conversion ratio; PRE = protein retention efficiency; HSI = hepatosomatic index; RGL = relative gut length. Data expressed as average (n=2 cages of 44 fish) ± SEM. Treatments within rows sharing superscript letters are not significantly different; p>0.05

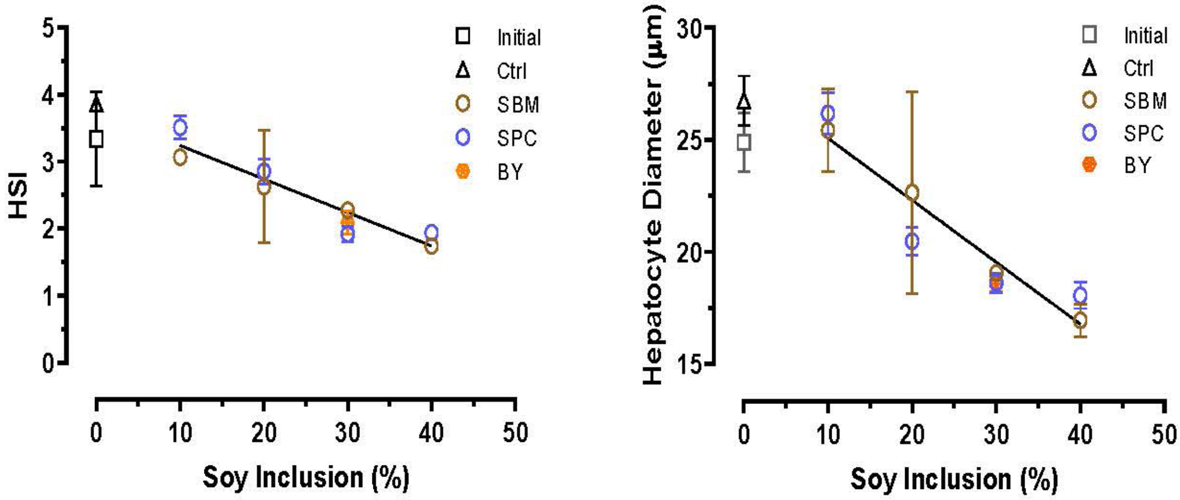
Diet	Control	SBM10	SBM20	SBM30	SBM40	SPC10	SPC20	SPC30	SPC40	BY20
Initial weight (g)	84.8±0.9	84.64±0.9	85.2±0.8	84.3±0.9	82.5±0.8	83.1±0.9	84.0±0.9	83.2±0.8	84.6±0.9	84.9±0.2
Final weight (g)	249.4±4.0^{a,b}	256.2±4.3^{a,b,c}	255.9±5.1^{a,b,c}	261.8±4.0^{b,c}	237.3±3.9^{a,b}	252.0±4.5^{a,b}	252.0±4.2^{a,b}	251.8±4.2^{a,b}	235.8±4.3^{a}	278.8±4.8^{c}
Weight gain (% BW)	194.0±4.9^{a,b,c}	202.7±3.1^{b,c}	200.4±0.8^{b,c}	210.4±2.2^{c,d}	187.5±1.9^{a,b}	203.2±3.1^{b,c}	200.2±4.1^{b,c}	202.7±1.7^{b,c}	178.9±3.7^{a}	227.1±4.8^{d}
SGR (% BW day^{-1})	1.86±0.03^{a,b,c}	1.91±0.02^{b,c}	1.90±0.002^{b,c}	1.95±0.01^{c,d}	1.82±0.01^{a,b}	1.91±0.02^{b,c}	1.90±0.01^{b,c}	1.91±0.01^{b,c}	1.77±0.02^{a}	2.04±0.03^{d}
Feed intake (g fish^{-1} day^{-1})	2.7±0.2^{a}	2.7±0.1^{a}	2.8±0.1^{a}	3.0±0.1^{a,b}	2.8±0.1^{a,b}	2.9±0.1^{a,b}	2.9±0.1^{a,b}	3.0 ± 0.1^{a,b}	3.0±0.1^{a,b}	3.3±0.3^{b}
FCR	0.94±0.02^{a}	0.93±0.02^{a}	0.95±0.01^{a,b}	0.98±0.003^{a,b}	1.05±0.002^{b,c}	0.98±0.01^{a,b}	0.99±0.02^{a,b}	1.03±0.0003^{a,b}	1.15±0.04^{c}	1.00±0.03^{a,b}
PRE	0.20±0.27^{a}	0.23±0.002^{a,b,c}	0.23±0.0002^{a,b,c}	0.24±0.01^{a,b}	0.23±0.003^{a,b,c}	0.23±0.01^{a,b,c}	0.24±0.01^{b,c}	0.23±0.003^{a,b,c}	0.20±0.01^{a}	0.26±0.01^{c}
HSI (%)	3.9±0.1^{c}	3.1±0.1^{a,b,c}	2.6±0.8^{a,b,c}	2.3±0.1^{a,b,c}	1.7±0.04^{a}	3.5±0.2^{b,c}	2.9±0.2^{a,b,c}	1.9±0.1^{a,b}	1.9±0.04^{a,b}	2.1±0.2^{a,b}
RGL (%)	1.5±0.1	1.6±0.04	1.4±0.1	1.3±0.1	1.5±0.2	1.5±0.04	1.5±0.1	1.5±0.02	1.8±0.03	1.5±0.2
Survival (%)	100	100	100	100	100	100	100	100	100	100

4.3.3.1.Histology

4.3.3.2.Liver

There was a strong negative correlation between soy inclusion level and hepatocyte diameter, supported by the concomitant decrease in HSI score (Figure 4.7). This response may reflect the capacity for soy proteins to reduce fat accumulation in the liver (Zhou, et al., 2014). Apart from hepatocyte size there were no other significant abnormalities observed. This correlation seems counter intuitive when considering other performance data (Table 9), i.e. with the exception of the 40% soy inclusion diets, the other diets performed equally as well, and in the case of the BY20 diet, better than the control group.

Figure 4.7. Increasing soy content of diets for gold spot grouper significantly decreases HSI ($y = 3.74-0.05x$; $r^2=0.697$) and hepatocyte size ($y = 27.84-0.28x$; $r^2=0.695$). Mean ± SEM; n=2.



4.3.3.3.Intestine

There was no significant correlation between villi length and diet treatment, while there was some evidence of mucous present in the lumen, dilated lumen and or mild multifocal leukocytes in the lamina propria and /or muscularis in some individual fish, although this was highly variable within treatment and most fish gut tissue were considered normal (Figure 4.9, Figure 4.10).

Figure 4.8. Liver histology of A. Initial, B. Control, C. SBM10, D. SBM20, E. SBM30, F. SBM40, G. SPC10, H. SPC20, I. SPC30, J. SPC40. Increasing soy content in diets for gold spot grouper significantly decreases hepatocyte size. Scale bar: 100μm

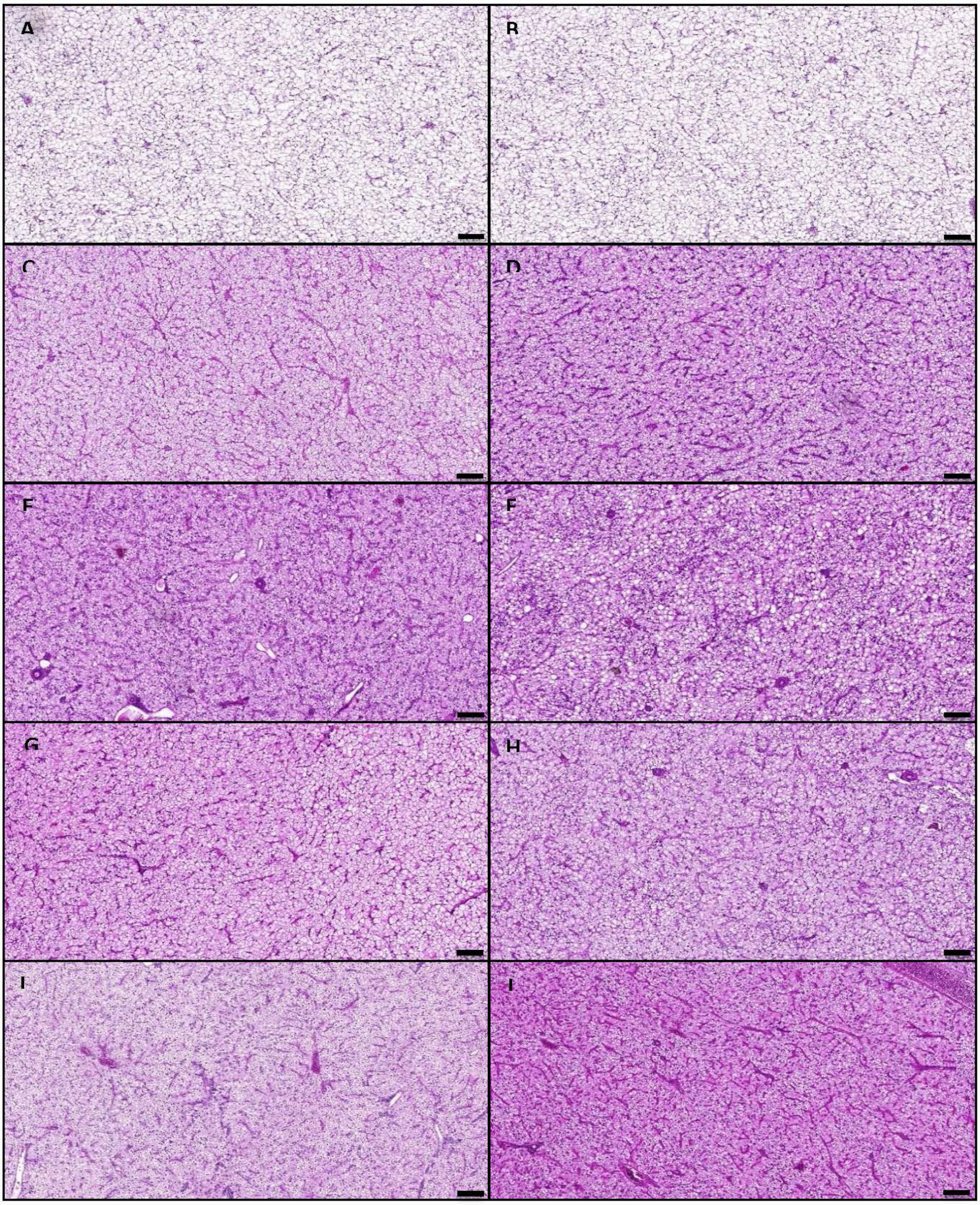


Figure 4.9. Control treatment hind gut cross section. Scale bar = 200µm.

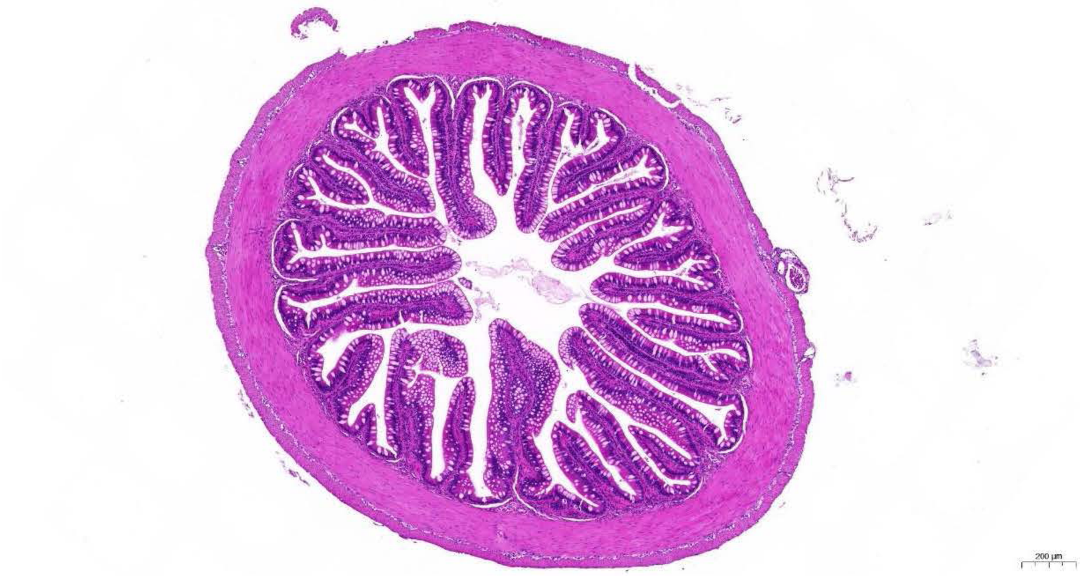


Figure 4.10. SPC40 treatment hind gut cross section. Scale bar = 200µm.

4.3.3.4.Pyloric Caeca

There was some mild lumen dilation in some sections of tissue and/or small multifocal areas of leukocytic infiltration in the submucosa in some of the individual fish sampled. Goblet cell prevalence was generally considered low to medium across all dietary treatments. There were no obvious pathologies or correlations with dietary treatment and the pyloric caeca was generally considered to be normal (Figure 4.11, Figure 4.12, Figure 4.13).

Figure 4.11. Control treatment pyloric caeca cross section. Scale bar = 1000µm

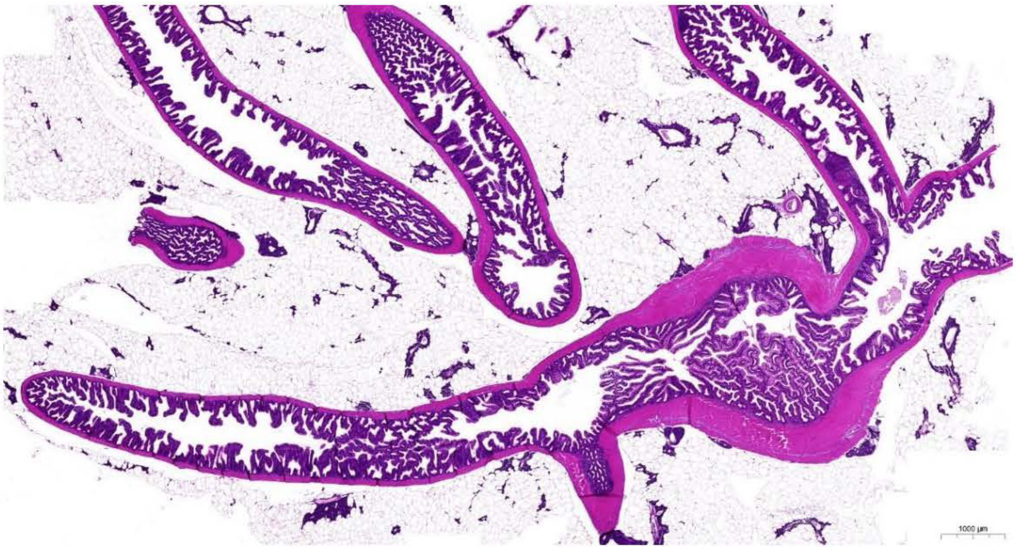


Figure 4.12. SBM40 treatment pyloric caeca cross section. Scale bar = 1000µm

4.3.4. Conclusion

Based on feed conversion and growth data gold spot grouper have a good capacity to utilize soy ingredients as protein sources to partially replace FM up to 30% inclusion in diets when diets are nutritionally balanced. Above this level there is a decline in growth rates and a decrease in feed conversion efficiency. However, the liver histology results suggest that the long-term health implications of feeding high soy diets to grouper should be considered. In the short term there seems to be a tradeoff between growth and liver health in terms of the utility of soy for grouper. The liver is a very robust organ therefore it may be possible to still achieve excellent growth rates during grow out using soy diets up to 30% inclusion, however longer term on-farm growth trials need to be undertaken to confirm this.

An encouraging result from this study was obtained from fish fed the BY20 diet, which performed better than the Control group. This diet contained 20% brewer's yeast and 30% SBM and would provide cost effective alternative protein sources to fishmeal. Growth on this diet was mainly driven by greater feed intake; however, there may potentially also be synergistic ingredient responses occurring. As with all of the soy diets there was a direct correlation between hepatocyte diameter and soy inclusion; therefore, long term growth studies should be considered to confirm the utility of these types of formulations.

4.4. Assessing Fishmeal Quality Interactions in Aquafeeds for Gold Spot Grouper

4.4.1. Introduction

One of the overall goals of this project is to formulate diets for grouper with optimal soybean meal (SBM) and soy protein concentrate (SPC) inclusion. The strategy is to reduce or replace other protein sources including fishmeal; however, if the fishmeal ingredient quality changes the "optimal" inclusion of the soy based ingredients is unlikely to remain constant, particularly if there is no change in the relative proportion of other protein sources in the diet. The previous study section 4.3 found that optimal inclusion of SBM or SPC in diets for gold spot grouper to promote growth were approximately 30% with 18% fishmeal. The fishmeal used in that study contained 65% crude protein and only SBM or SPC, not a combination of both, were tested in diets. The fishmeal crude protein content used in manufacturing commercial marine fish or grouper diets in SE Asia is typically ~65% CP with lesser quality or fish by-product meals at ~60% CP or less. A premium grade fishmeal will normally contain >70% CP.

There are many studies that have tested the effect of fishmeal replacement with soy proteins, particularly so for non-grouper species (e.g. Bowyer, et al., 2013b; Zhou, et al., 2005); however, there is scant work considering the interactive effects of fishmeal quality and SBM and/or SPC inclusion and its impact on fish growth. Understanding these interactions will provide information that will facilitate more accurate feed formulations for grouper. The aim of this study was to test flexibility of feed formulation for gold spot grouper given different quality fishmeal and soy ingredients.

4.4.2. Material and Methods

This study was conducted at PSFI. The experiment design evaluated two types of fishmeal; a premium quality fishmeal (Peruvian anchovy; 75% CP) and a lower quality fishmeal byproduct meal (Tuna trimmings; 65% CP) and two soy protein products; a solvent extracted SBM (Argentinian origin) and a SPC. There were eight diet treatments formulated: a premium fishmeal control (FM-P Ctrl), a fishmeal byproduct control (FM-B Ctrl), a 30% inclusion of either SBM (SBM30) or SPC (SPC30) in each of the fishmeal controls and a blend of 15% SBM + 15% SPC (SBMSPC) in each of the fishmeal controls. Formulations are presented in Table 4.14.

All diets were formulated to be isonitrogenous (49.5% CP), isocalorific (20.7 MJ/kg gross energy) and isolipidic (12.3% fat) (Table 15). The diets were formulated to precisely balance all essential amino acids (Table 4.15; Figure 4.13). Diets also contained 0.1% (DM) of Y_2O_3 inert marker to determine diet digestibility at the conclusion of the trial.

Ten grouper (mean±sem = 499.8±0.3 g fish^{-1}) were each stocked into three replicate 200

L cages per diet treatment and held at (mean±stdev) 28.2±0.6 °C in a saltwater recirculating aquaculture system: salinity 32.3±0.8 ppt; $NH3/4^+$ 0.25±0.1; pH 7.7±0.2; DO 6.9±0.3 mg l^{-1}. Fish were hand fed to satiation once daily for eight weeks.

Performances were assessed based on growth and feed conversion indices:
Feed intake (g/fish) = (Total feed given-Total uneaten feed)/(Fish number)
Weight gain (%) = Final fish weight-Initial fish weight/ Initial fish weight x 100
Feed Conversion Ratio=Weight of consumed food/weight gain
Protein Efficiency Ratio (PER) = Fish weight gain/ protein consumed
Specific growth rate (% per day)=[(ln final weight-ln initial weight)/days] x 100

Carcass compositional analyses were also undertaken of an initial sample and final subsample of five fish from each replicate to test if the proximate composition (crude protein, fat, ash and gross energy) of gold spot grouper was influenced by the experiment diets.

Nutrient digestibility's of the diets were also assessed at the conclusion of the study using the same techniques presented in Section 4.1 and calculated using Eq.2 and Eq.3.

4.4.2.1.Data Analyses

Data was statistically compared using NCSS-8.0.23 after assumptions on the normality and homogeneity of data were met. ANOVA and Tukey-Kramer post-hoc multiple comparison tests were considered significant at $p<0.05$.

4.4.3. Results and Discussion

There was 100% survival at the conclusion of the trial. Growth and FCRs were excellent across all diet treatments. On average, grouper increased in body size by 87% growing from approximately 500 g to just under 1 kg in eight weeks. FCR's were approximately 1.1:1 on average. There were no significant differences among diet treatments with respect to growth, feed intake or feed conversion efficiencies (Table 4.16). Average whole fish wet weight composition for moisture (67.7%), ash (5.0%), protein (17.7%), fat (8.2%) and gross energy (7.4 MJ kg^{-1}) did not vary significantly among treatment groups (Table 4.17). Digestibility of all of the diets was good and comparable with the previous trial section 4.3 and did not vary significantly for dry matter (70.8%), protein (89.8%), fat (93.0%), organic matter (80.6%), NFE (56.5%) or energy (83.2%) (Table 4.18).

Fishmeal can be supplemented from almost 50% of a diet to a low of approximately 15% or 18%, depending on the protein content, using up to 30% SBM and/or SPC. SBM and SPC can be supplied as separate ingredients or as a blend without negatively affecting growth and FCR of gold spot grouper. However, it is important to acknowledge the effect on hepatocytes and HSI of gold spot grouper as noted in the previous study section 4.3 and longer term growth trials to determine health impacts are recommended.

There are many studies that have assessed the utilization of alternative plant protein sources in aquafeeds over the past 20+ years (see reviews by Daniel, 2018; Enami, 2011; Gatlin, et al., 2007; Kumar, et al., 2014) and there is great potential to formulate cheap, nutritionally appropriate diets for grouper with very low or potentially zero fishmeal. This requires a sound knowledge of nutritional requirements; however, for grouper there are still key areas where quantitative data is lacking. This includes amino acid, fatty acid requirement and micronutrient (vitamin and mineral) requirements.

4.4.4. Conclusion

The results from this study clearly demonstrate that there is good potential for flexibility in feed formulations for gold spot grouper, irrespective of the protein content of fishmeal; provided diets are formulated to be nutritionally balanced. This requires information on the nutrient profile of the raw ingredients, as well as an understanding of the nutritional requirements of the species. This concept should be transferable to other closely related *Epinephelus spp.* longer term studies are recommended to assess the health impacts of low fishmeal diets for grouper.

Table 4.14. Diet formulations (% Dry matter basis). FM-P = fishmeal premium; FM-B = Fishmeal byproduct

Ingredients	FM-P Ctrl	FM-B Ctrl	FM-P SBM30	FM-B SBM30	FM-P SPC30	FM-B SPC30	FM-P SBMSPC	FM-B SBMSPC
Blood meal	2.00	6.28	6.58	6.74	6.39	6.86	8.00	8.00
Brewer's Yeast	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Choline Chloride (70%)	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Corn Gluten	0.64	1.66	5.50	5.50	0.60	0.60	0.60	0.60
Diatom. Earth	5.95	0.55	2.75	2.74	6.38	4.62	2.29	0.00
Fish Oil	3.50	2.58	4.70	4.56	6.39	5.83	5.14	4.55
Fishmeal Premium	46.00	0.00	15.00	0.00	15.00	0.00	15.00	0.00
Fishmeal By-product	0.00	48.00	0.00	18.00	0.00	18.00	0.00	18.00
Krill Meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Maize Starch	9.50	9.50	7.02	5.47	9.55	9.55	9.55	9.50
Methionine	0.13	0.22	0.50	0.50	0.50	0.44	0.50	0.50
Poultry meal	8.50	8.00	8.00	8.00	5.00	5.00	5.00	5.00
Rovimix Stay-C	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
SBM	0.00	0.00	30.00	30.00	0.00	0.00	15.00	15.00
SPC	0.00	0.00	0.00	0.00	30.00	30.00	15.00	15.00
Soy Lecithin	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Taurine	0.77	0.84	1.00	1.00	1.00	1.00	1.00	1.00
Vit-min premix^	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Wheat Flour	10.21	9.57	6.15	4.69	6.39	5.30	10.12	10.05
NaH_2PO_4	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Y_2O_3	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10

^ Ridley Corporation Ltd; marine fish vitamin & mineral premix, formulation undisclosed

Table 4.15. Compositional analysis (DM basis) of key raw ingredients and experiment diets

	Raw Ingredients				Diets							
Nutrient (%)	**FM-P**	**FM-B**	**SBM**	**SPC**	**FM-P Ctrl**	**FM-B Ctrl**	**FM-P SBM30**	**FM-B SBM30**	**FM-P SPC30**	**FM-B SPC30**	**FM-P SBMSPC**	**FM-B SBMSPC**
Dry Matter	92.8	94.3	87.6	91.6	94.3	94.6	95.3	95.1	94.9	93.3	92.6	93.7
Protein	74.7	65.5	54.0	72.5	49.7	50.2	49.6	49.8	49.6	49.9	48.0	49.0
Fat	10.2	9.4	2.6	1.2	13.0	11.3	13.8	13.4	11.9	11.6	12.0	11.6
Ash	14.6	22.5	6.8	6.2	15.6	14.6	10.1	12.1	13.3	13.5	9.3	8.7
NFE*	0.4	2.7	36.6	20.1	21.7	23.9	26.5	24.8	25.2	25.0	30.7	30.7
GE (MJ/kg)	21.4	19.4	19.5	20.3	20.3	20.4	21.5	21.0	20.3	20.3	21.1	21.0
NFE	63.5	55.4	37.1	53.4	42.5	41.1	42.0	42.1	41.2	41.2	38.9	39.6
Alanine	4.9	4.6	2.3	3.1	3.1	3.5	2.9	3.1	2.8	2.9	2.7	2.8
Arginine	4.0	3.9	3.9	5.3	2.6	2.7	2.7	2.8	2.9	3.1	2.8	2.9
Aspartic acid	6.1	6.0	6.3	8.4	4.0	4.4	4.5	4.6	4.8	5.0	4.7	4.8
Cysteine	0.5	0.5	0.8	0.9	0.4	0.4	0.5	0.5	0.5	0.5	0.5	0.5
Glutamic acid	9.2	8.2	10.3	13.7	6.4	6.4	7.3	7.5	7.5	7.6	7.3	7.4
Glycine	4.7	4.4	2.2	3.0	3.0	3.1	2.4	2.6	2.4	2.5	2.4	2.4
Histidine	2.3	2.1	1.4	1.8	1.4	1.6	1.4	1.4	1.4	1.5	1.4	1.5
Isoleucine	2.9	2.8	2.1	3.0	1.8	1.9	1.8	1.9	1.9	2.0	1.8	1.8
Leucine	4.9	4.9	3.9	5.5	3.5	4.0	4.0	4.3	3.9	4.0	3.9	4.0
Lysine	5.1	5.0	3.2	4.5	3.1	3.5	3.0	3.1	3.2	3.3	3.1	3.2
Methionine	1.8	1.7	0.8	1.0	1.2	1.3	1.4	1.4	1.3	1.3	1.3	1.3
Phenylalanine	2.7	2.7	2.7	3.6	2.0	2.3	2.4	2.5	2.3	2.5	2.3	2.5
Proline	2.8	3.1	3.1	4.8	2.2	2.2	2.2	2.4	2.1	2.3	2.3	2.3
Serine	2.4	2.6	3.0	4.0	1.8	2.0	2.0	2.2	2.0	2.2	2.1	2.2
Threonine	2.7	2.8	2.1	2.9	1.8	2.1	1.8	1.9	1.9	2.0	1.9	2.0
Tyrosine	2.3	2.3	2.0	2.4	1.7	1.7	1.8	1.9	1.7	1.7	1.7	1.7
Valine	4.1	4.0	2.2	3.0	2.9	3.3	3.1	3.3	3.2	3.3	3.1	3.1
Taurine	0.9	0.3	0.0	0.0	1.1	1.0	1.1	1.1	1.3	0.8	1.1	1.0

*NFE Calculated by difference: 100 – (protein + fat + ash)

Figure 4.13. Amino acid profile (% diet; dry matter basis as measured) of the eight diets used in the fishmeal quality interaction study for gold spot grouper

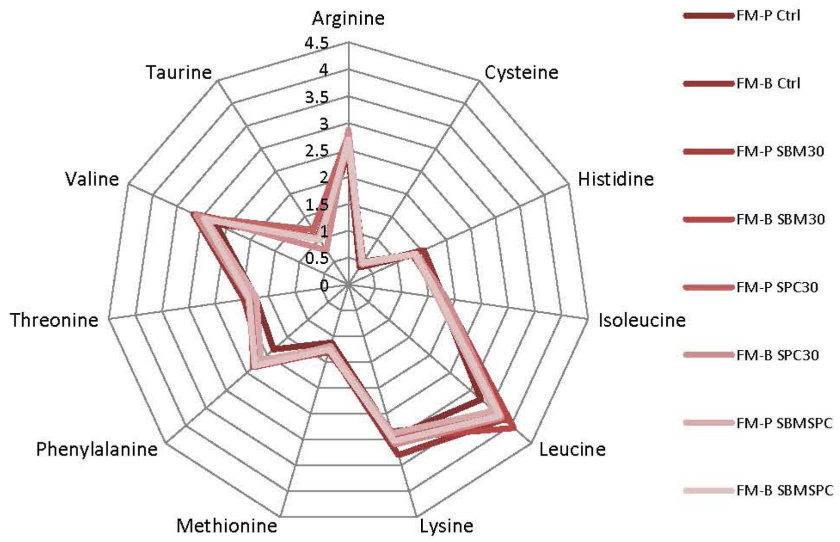


Table 4.16. Performance of gold spot grouper (mean +/- SE; n=3) fed diets with varying fishmeal ingredient quality and soy protein form. No significant differences were found among all diets within each parameter (*P>0.05*)

Parameter	FM-P Ctrl	FM-B Ctrl	FM-P SBM30	FM-B SBM30	FM-P SPC30	FM-B SPC30	FM-P SBMSPC	FM-B SBMSPC
Initial weight (g)	499.3±0.68	499.8±1.07	501.4±0.72	499.1±1.19	499.4±0.35	499.5±1.03	499.9±0.70	499.7±0.69
Final weight (g)	936.2±20.0	937.1±33.8	978.2±35.2	912.9±37.1	943.5±24.0	915.3±15.6	927.7±29.5	923.2±44.8
Weight gain (% BW)	87.33±0.04	87.47±0.06	95.62±0.07	82.92±0.08	88.96±0.05	83.68±0.03	85.03±0.06	84.54±0.09
Weight gain (g fish^{-1} day^{-1})	6.73±0.51	6.42±0.24	7.36±0.54	6.56±0.46	6.37±0.58	6.50±0.70	6.71±0.32	6.83±0.36
SGR (% BW day^{-1})	0.90±0.01	0.91±0.03	0.97±0.06	0.99±0.05	1.01±0.06	1.03±0.01	0.89±0.04	0.98±0.05
Feed intake (g fish^{-1} day^{-1})	7.61±0.55	7.66±0.35	8.39±0.57	7.39±0.28	7.11±0.45	7.33±0.60	7.63±0.62	7.39±0.35
FCR (g g^{-1})	1.13±0.01	1.19±0.01	1.14±0.02	1.13±0.04	1.12±0.03	1.13±0.05	1.13±0.05	1.08±0.02
Survival (%)	100	100	100	100	100	100	100	100

Table 4.17. Average whole body proximate composition (+/- se; n=3; wet weight basis) of gold spot grouper fed each of the test diets. FM-P = fishmeal premium; FM-B = Fishmeal byproduct. No significant differences were found among diets within each parameter (p>0.05)

	Initial	FM-P Ctrl	FM-B Ctrl	FM-P SBM30	FM-P SPC30	FM-B SBM30	FM-B SPC30	FM-P SBMSPC	FM-B SBMSPC
Moisture (%)	65.1	68.6±0.6	70.2±1.2	66.8±0.4	68.5±0.6	66.3±0.6	67.3±1.0	68.0±1.1	66.0±0.6
Ash (%)	5.6	5.1±0.1	4.0±0.2	5.4±0.2	4.5±0.2	4.6±0.3	5.1±0.2	5.2±0.6	5.6±0.04
Protein (%)	18.7	16.9±0.5	16.6±0.7	18.2±0.6	17.7±0.2	18.7±0.5	18.2±0.8	17.4±0.8	18.2±0.3
Fat (%)	8.5	7.9±0.4	7.8±0.5	8.6±0.2	8.0±0.1	9.0±0.2	8.6±0.4	7.8±0.5	8.2±0.4
Energy (MJ/kg)	7.9	7.1±0.3	7.1±0.4	7.5±0.1	7.4±0.2	7.9±0.2	7.6±0.2	7.2±0.4	7.5±0.3

Table 4.18. Apparent diet digestibility coefficients (mean +/- se; n=3). Dry Matter (DM); Organic matter (OM); Nitrogen free extract (NFE). FM-P = fishmeal premium; FM-B = Fishmeal byproduct. No significant differences were found among diets within each parameter (P>0.05)

Parameter	FM-P Ctrl	FM-B Ctrl	FM-P SBM30	FM-P SPC30	FM-B SBM30	FM-B SPC30	FM-P SBMSPC	FM-B SBMSPC
DM	0.67±0.02	0.74±0.01	0.73±0.01	0.69±0.02	0.70±0.02	0.70±0.01	0.70±0.01	0.72±0.01
Protein	0.83±0.01	0.90±0.01	0.91±0.01	0.91±0.01	0.91±0.01	0.92±0.01	0.89±0.002	0.91±0.001
Fat	0.92±0.00	0.91±0.01	0.94±0.01	0.94±0.003	0.92±0.01	0.95±0.002	0.94±0.01	0.93±0.01
OM	0.79±0.02	0.83±0.01	0.82±0.01	0.80±0.02	0.81±0.01	0.81±0.01	0.78±0.01	0.80±0.004
NFE	0.61±0.05	0.66±0.02	0.58±0.02	0.48±0.04	0.54±0.03	0.52±0.02	0.55±0.01	0.58±0.01
Energy	0.81±0.02	0.85±0.01	0.84±0.00	0.83±0.01	0.83±0.01	0.84±0.01	0.81±0.01	0.83±0.00

4.5. Optimal Feeding Frequency for Gold Spot Grouper

4.5.1. Introduction

Feeds and feeding account for the largest proportion of aquaculture production costs, often accounting for over 50% of operation budgets (Rana, et al., 2009). Implementing practical and effective feed management strategies optimizing how often stock should be fed can significantly reduce these costs; however, optimal feeding regimes can vary considerably from species to species (e.g. Al Zahrani, et al., 2013; Wang, et al., 2007). While gold spot grouper is regarded as a valuable aquaculture marine fish, there is surprisingly little information available identifying optimal feeding regimes for this species. The aim of this study was to identify an optimal feed frequency for gold spot grouper to promote good growth and feed conversion efficiency.

4.5.2. Material and Methods

The design considered four feed frequency levels representing a practically achievable regime based around typical farm working hours. The feed frequency treatments were: one 8am feed, one 4pm feed, two feeds per day (8am and 4pm), or three feeds per day

(8am, 12pm, 4pm). 35 gold spot grouper (approx. 15 g $fish^{-1}$) were each stocked into three replicate 200 l cages and held at 28°C in a saltwater recirculating aquaculture system for six weeks. At each feeding event fish were fed to apparent satiation with a 6mm commercial marine fish floating pellet; 50% crude protein, 14% crude fat. Fish were fasted for 48 h prior to final sampling.

Performance was assessed based on growth and feed conversion parameters which included:

Weight gain (%) = Final body weight – initial body weight / initial body weight x 100
Daily weight gain (g $fish^{-1}$ day^{-1}) = Final body weight – initial body weight / number of days
Specific Growth Rate (SGR) = ((LN (final weight)-LN (initial weight)) / number of days x 100
Food Conversion Ratio (FCR) = Weight of consumed food / weight gain

All data were compared statistically using one-way ANOVA followed by Tukeys post hoc test on significant terms; $p<0.05$.

4.5.3. Results and Discussion

There were zero mortalities throughout the trial. FCR's at all feed frequencies were excellent at <1. Weight gain (g $fish^{-1}$ day^{-1}) for fish fed 3x daily (1.17±0.06) or 1x 4pm feed (1.16±0.01) were significantly higher than fish fed 1x 8AM feed (0.69±0.01) or 2x daily (0.98±0.03). Feed intake (g $fish^{-1}$ day^{-1}) for fish fed 8AM feed was significantly lower, ranging from 38% - 58%, than other frequencies (Table 4.19). Fish increased body weight on average between 180% (8AM feed) to 332% (3x daily feed) in body weight over 6 weeks. Fish in the single 4pm feed treatment increased in body weight by an average of 325%.

Fish exhibit strong circadian rhythms naturally and these can persist in culture (Kadri, et al., 1991). Observations of grouper behavior during the trial, and also of the remaining stock held in the general population, indicate a clear reluctance for fish to feed in the morning, with fish tending to aggregate at the bottom of cages or tanks. This behavior likely resulted in the relatively slower growth of grouper in this treatment group (Table 4.19). The timeframe of this study was sufficient to demonstrate a statistically significant result favoring afternoon feeding. The morning fed group may have, over time, become acclimated to the morning feeding regime (Reebs, 2002); however, this group would always have been behind in body weight compared to those fed initially in the afternoon, which were effectively given a "head start" by applying a feed frequency regime complementing this species apparent feeding rhythm.

Gold spot in this study did not feed at a consistent daily rate over the course of the study, which is typical of most fish species (Boujard and Leatherland, 1992). Gold spot would generally consume less feed following a day where a relatively higher amount of feed was consumed (Figure 4.14); fish would often gorge one day and would still have full and distended stomachs the following day (pers. obs.). This could imply that a day might be skipped without feeding without significantly impacting on growth further reducing feed input costs; however, this remains to be tested.

The results of this study indicate that a single afternoon feed is suitable for gold spot grouper; fish fed a single feed once per day in the afternoon performed as well as those fed either twice or three times daily, and significantly better than those fed only in the morning (Table 1.1). Further, the feed intake data indicates that, for fish of the size used in this study, satiated feeding is approximately equivalent to 3 – 3.5% of body weight per day (based on the geometric mean body weight of initial and final weights). This implies that, regardless of a single afternoon or multiple feeds, once gold spot grouper are satiated they will not (or cannot) consume much more. This can be seen in Table 1.1 as no significant difference among the 2x, 3x or pm feed groups with regard to feed intake.

4.5.4. Conclusion

The results of this study have clear implications for the feed management of gold spot grouper; a single afternoon feed is sufficient to elicit a good growth and feed conversion response. This can potentially reduce labor and feed costs associated with feeding and feed management. The fish used in this study were <100g, ontogenetic shifts in feeding behavior could occur, particularly as this is a large growing animal which would naturally occupy a variety of trophic levels throughout its life cycle, therefore it would be prudent to also consider optimal feed frequency with different size gold spot grouper.

Table 4.19. Performance of gold spot grouper (mean +/- SE) fed one of four daily feeding frequencies. AM = one 8AM feed only; 2X = one 8AM feed + one 4pm feed; 3X = 8AM feed, 12pm feed, 4pm feed; PM = 4pm feed only. Treatments within rows sharing superscript letters are not significantly different; p>0.05

Performance Parameter	AM	2X	3X	PM
Initial weight (g)	14.77 ± 0.19	14.79 ± 0.15	14.52 ± 0.29	14.61 ± 0.18
Final weight (g)	43.77 ± 0.43^{a}	56.14 ± 1.14^{b}	63.72 ± 2.65^{c}	63.19 ± 0.50^{c}
Weight gain (% BW)	196.4 ± 5.61^{a}	279.8 ± 11.39^{b}	338.4 ± 9.54^{c}	332.5 ± 2.47^{c}
Weight gain (g fish -1 day -1)	0.66 ± 0.01^{a}	0.94 ± 0.03^{b}	1.12 ± 0.06^{c}	1.11 ± 0.01^{c}
SGR (% BW day -1)	2.47 ± 0.05^{a}	3.03 ± 0.07^{b}	3.36 ± 0.05^{c}	3.33 ± 0.01^{c}
Feed intake (g fish -1 day -1)	0.68 ± 0.01^{a}	0.94 ± 0.03^{b}	1.08 ± 0.08^{b}	0.96 ± 0.03^{b}
FCR (g g-1)	0.98 ± 0.04^{a}	$0.96 \pm 0.03^{a,b}$	$0.92 \pm 0.03^{a,b}$	0.83 ± 0.02^{b}
Survival (%)	100	100	100	100

4.6. Optimal Stocking Density for Gold Spot Grouper

4.6.1. Introduction

Optimizing stocking density of fish in culture can lead to significantly improved growth and feed conversion efficiencies (Ellis, et al., 2002; Pirozzi, et al., 2009). While the importance of stocking density in grouper aquaculture is understood, at least in general terms (Rimmer, et al., 2004), there is little published information describing optimal densities for gold spot grouper in commercial culture. Of the studies that have been conducted on this species, only larvae (Duray, et al., 1997) or small fingerlings (Hseu, 2002) have been considered. The aim of this study was to identify the optimal stocking density of large (approx. 300 g) gold spot grouper that will promote good growth and feed conversion efficiency.

4.6.2. Material and Methods

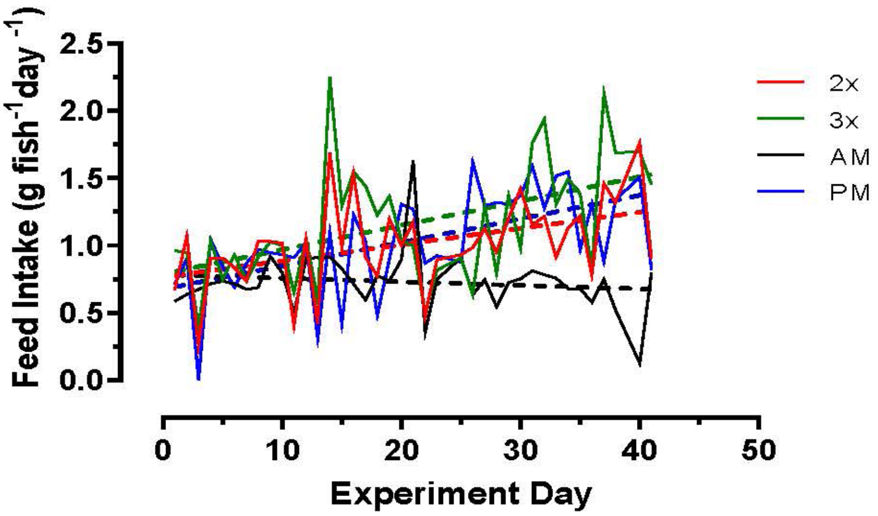
The experiment evaluated four stocking densities; 4, 10, 20 or 30 gold spot grouper (initial body weight ~300 g) stocked in to three replicate 200 L cages per density treatment held within a 10,000 L tank at 28°C in a saltwater recirculating aquaculture system for 8 weeks. Initial densities equated to approximately 6, 15, 30 or 45 kg m^{-3}. Fish were fed a 6mm commercial marine fish floating pellet; 50% crude protein, 14% crude fat.

Performance was assessed based on growth and feed conversion parameters which included:

- Weight gain (%) = Final body weight – initial body weight / initial body weight x 100
- Daily weight gain (g $fish^{-1}$ day^{-1}) = Final body weight – initial body weight / number of days
- Specific Growth Rate (SGR) = ((LN (final weight)-LN (initial weight)) / number of days x 100
- Food Conversion Ratio (FCR) = Weight of consumed food / weight gain

All data were compared statistically using one-way ANOVA followed by Tukeys post hoc test on significant terms; $p<0.05$. Nonlinear regression analysis was used to determine the optimal initial stocking density.

Figure 4.14. Average daily feed intake (g $fish^{-1}$; n=3) of gold spot grouper for: 2x, twice daily feeding; 3x, three times daily feeding; Am one time morning feed, or PM; one time afternoon feed. Zero intake on Day 3 indicates a storm or weather event where fish would not feed. Average data shown without standard error for clarity. Dashed linear regression lines indicate general trend of feed consumption over time. Fasting days prior to sampling not included in this data set



4.6.3. Results and Discussion

There were zero mortalities at the conclusion of the trial. Final stocking densities equated to 10.0, 30.3, 59.0 and 90.8 kg m^{-3} (Table 4.20). There was no significant difference among the three highest densities in growth rate with the lowest density the worst performing. FCR's were also excellent (<1.0) for the three highest density groups with the lowest density group demonstrating relatively poorer FCR (1.04), although these were not statistically different. The three highest density groups put on an average of 98.9% in body weight over 8 weeks while the lowest density increased in biomass by an average of 65%. The results indicate that gold spot grouper can be successfully cultured at high densities provided adequate nutrition and good water quality are provided. Adequate oxygen saturation will become the first limiting condition at high densities therefore it is critical that this parameter is monitored regularly.

The highest density at the conclusion of the trial was approximately 90 kg m^{-3}. This was not significantly different from the second and third level densities when considering growth and feed efficiency (Table 4.20) which may imply that even higher stocking densities may be achieved without compromising growth. However, when considering nonlinear regression analyses of the data (Figure 4.15), an optimal initial stocking density of 33.6 kg m^{-3} is identified. High stocking densities of up to 100 kg m^{-3} have been achieved for salmon at 9.3°C (Calabrese, et al., 2017), the culture of tropical species such as grouper in higher water temperatures would severely limit upper stocking thresholds without adequate O_2 input. It is important to consider that the data and conclusions reported in this study are based on growth and feed intake data only. While general observations of the health status of the fish indicated that they were in good condition, i.e. zero mortalities, good feed intake and growth and no obvious signs of physical trauma such as fin and/or eye damage, the measurement of blood parameters such as cortisol, pCO_2 and plasma pH would indicate conclusively the welfare status of the fish. Cannibalism was often observed in the general population of gold spot grouper in holding tanks at the research facility; however, this behavior was never observed during this trial nor indeed in any of the trials documented in this report. This is likely due to the close grading of fish size for experiments at stocking.

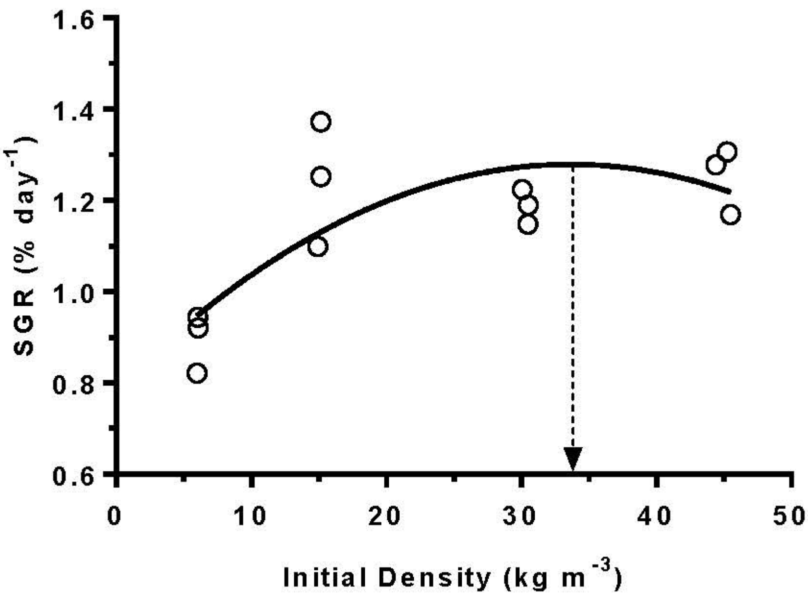
4.6.4. Conclusion

From the results of this study it is evident that gold spot grouper can tolerate high stocking densities while still maintaining good growth rates and feed conversion efficiencies. At the lowest density growth and feed conversion were relatively poor. Fish at the low density were skittish and reluctant to feed. This has practical implications for both farm management practices as well as consideration for appropriate numbers of fish to use when running feed trials with this species if optimal growth rates are desired.

Table 4.20. Performance of gold spot grouper (mean +/- SE; n=3) stocked at four different densities in 200 l cages. Treatments within rows sharing superscript letters are not significantly different; p>0.05

	Fish per Cage			
Parameter	**4**	**10**	**20**	**30**
Initial Density (kg m^{-3})	6.03 ± 0.03	15.07 ± 0.08	30.36 ± 0.15	45.04 ± 0.33
Final Density (kg m^{-3})	9.97 ± 0.25	30.27 ± 1.47	59.02 ± 0.51	90.78 ± 1.88
Initial weight (g)	301.7 ± 3.90	301.5 ± 2.91	303.6 ± 2.38	300.3 ± 1.83
Final weight (g)	498.3 ± 7.15^a	605.5 ± 12.49^b	590.2 ± 10.49^b	605.2 ± 7.72^b
Weight gain (% BW)	65.16^a	100.83^b	94.4^b	101.53^b
Weight gain (g fish^{-1} day^{-1})	3.51 ± 0.21^a	5.43 ± 0.53^b	5.12 ± 0.12^b	5.45 ± 0.25^b
SGR (% BW day^{-1})	0.89 ± 0.04^a	1.24 ± 0.08^b	1.19 ± 0.02^b	1.25 ± 0.07^b
Feed intake (g fish^{-1} day^{-1})	3.88 ± 0.53^a	5.11 ± 0.48^b	4.87 ±0.03^b	4.96 ± 0.19^b
FCR (g g^{-1})	1.04 ± 0.09^a	0.89 ± 0.002^b	0.90 ± 0.01^b	0.86 ± 0.02^b
Survival (%)	100	100	100	100

Figure 4.15. Relationship between specific growth rate and initial stocking density of gold spot grouper grown for eight weeks with an initial body weight of 300 g. SGR = 0.78 + 0.029x - 0.0004x^2 (r^2= 0.59). Vertex indicated at 33.6 kg m^{-3}.



5. Theoretical SBM and SPC Aquafeed Formulations for Grouper Growing To 1kg

The feed trials presented in this report demonstrate that there is considerable potential for flexibility in feed formulations for grouper, particularly with the utilization of soy proteins and different quality fishmeal's, This view is based on the caveat that diets are balanced for nutrient and energy requirements. There are however several assumptions that must be made with regard to feed formulations for grouper as there still remains fundamental areas that are very much understudied for grouper nutrition. This includes requirement studies for fish larger than 200 g, lipid nutrition and micronutrient (vitamin and mineral) requirements. There are several studies that have evaluated soy proteins and other raw materials in diets for grouper (e.g. Millamena, 2002; Shapawi, et al., 2013; Wang, et al., 2017); however, most of these studies are short term of several weeks duration.

There are virtually no studies that have investigated the long-term grow out performance of grouper with fishmeal replacement diets, particularly with soy proteins. While the results presented in section 4.3 indicate good potential for using a large proportion of brewer's yeast (20%) in diets for grouper, further work is required to assess the suitability of this protein at relatively high inclusion for long term feeding. The diets presented below are formulated for consideration for long-term grow out trials for grouper using practical, commonly used feed ingredients and focusing on the inclusion of soy proteins.

5.1. Approach to Diet Formulations

Section 4.1 determined the requirements for DP and DE for tiger grouper growing to 1 kg. Table 3.6 suggested a dietary specification for grouper at different growth stanzas. These diet specifications were chosen to achieve a balance between meeting the theoretical requirement for protein and energy for grouper and the practical ability to formulate diets on a digestible basis using a basic suite of raw ingredients. Raw ingredient ADC's are not available for most of the ingredients used in the formulations therefore an ADC value of 0.85 for gross energy and 0.9 for crude protein were cautiously assumed; this approximates the diet ADC's evaluated in sections 4.2, 4.3 and 4.4 above. In the absence of published species specific data, the nutritional model developed for tiger grouper in Section 4.1 and SBM and SPC tolerances established for gold spot grouper in sections 4.3 and 4.4 are assumed to be interchangeable for the exercise of formulating the theoretical diets presented in this section. Recent data published for gold spot grouper growing from 100-200 g (Yan, et al., 2020) indicates a dietary crude protein requirement of 46.7% identified using a broken-line model. Reanalysis of the data using non-linear regression identifies a requirement of 51.4% CP, this value being very similar to that estimated in Table 3.6. The application of broken line regression tends to identify threshold requirement (Shearer, 2000), which may of course be entirely appropriate, provided however that intake is maximized if feeding this type of diet. The lipid content of diets was chosen to provide appropriate levels of n3 fatty acids and based on published data indicating a requirement ranging from approximately 9-15% crude fat for small grouper <10 g initial body weight of different *Epinephelus spp.* (Lin and Shiau, 2003; Luo, et al., 2005c; Rahimnejad, et al., 2015; Tuan and Williams, 2007; Yoshii, et al., 2010).

Table 5.2 presents theoretical formulations using practical ingredients to meet the requirement of DP and DE at three different growth stanzas. Table 5.3 presents the theoretical nutrient composition of the diets and Table 5.4 presents the proximate and amino acid composition of the main ingredients used in the diet formulation. All diets were formulated on a dry matter basis. Soy protein inclusion was approximately 30% in all diets and chosen based on the good growth and protein retention achieved in the feed trials presented in sections 4.3 and 4.4. Additionally, each of the suite of soy protein diets were formulated with one of two quality fishmeal's; either a 55% crude protein or a 65% crude protein content. While relatively good growth was demonstrated at ~30% inclusion with soy proteins (section 4.3) it should be noted that after 8 weeks the liver histology indicated a negative correlation of soy inclusion with hepatocyte size and overall HSI; the long-term effects on the growth and health of grouper consuming relatively high soy diets is unknown.

6. Recommendations

A bioenergetic nutritional model has been developed describing the requirements for DP and DE for tiger grouper growing to 1kg. This represents the first application of this approach to this species and is a useful first step towards providing a knowledge foundation from which to develop appropriate diet formulations for different size classes of tiger grouper. However model predictions must be considered objectively within the context of the data sets used to derive key model parameters. For example, the growth model was developed mainly from data collated from our laboratory trials and conducted at 28 °C therefore to improve the predictive ability for on farm conditions a more comprehensive data set beyond those presented in this report is recommended including a range of temperatures encountered in grouper culture and relative for different fish sizes throughout grow out.

The studies in this report on the utilization of SBM and SPC as well as different fishmeal types demonstrated that grouper can effectively utilize different protein sources indicating that there is good potential for flexibility in feed formulations provided diets can be nutritionally balanced. A simple diet including 30% SBM and 20% brewer's yeast was shown to promote a very good intake and growth response in gold spot grouper. However long-term growth and health assessment on such diets, particularly considering the liver histology results which showed a negative correlation between soy inclusion and hepatocyte size is required.

There is a dearth of information on the nutritional requirements of grouper >200g. Some of the feed trials in this report sought to address this issue, particularly with the development of a factorial model for tiger grouper. Future studies should consider different fish sizes representative of grow out through to market size.

While there are now a number of publications available for grouper describing the fundamental nutritional requirements there remains key areas that are understudied. This includes amino acid requirements, and in particular, sulfur amino acid requirements as methionine is often the first limiting amino acid when replacing animal proteins with plant proteins, lipid nutrition including fatty acid requirements and micronutrient requirements for vitamins and minerals.

This report focused on two *Epinephelus* species, tiger and gold spot grouper. Nutritional requirements may be species specific however in the absence of any other available data some assumptions need to be made with regards to decisions around appropriate dietary specifications. For different species within the same genus and which occupy a similar trophic level one may be considered a surrogate for the other until more accurate species-specific data becomes available.

Hybrid grouper (tiger x giant) aquaculture is becoming more widespread and therefore this necessitates a need for focused nutrition research including an assessment of the utilization of soy protein in diets.

Table 5.1. Practical diet formulation of tiger grouper growing from approximately 10-1000 g. Gross energy and crude protein values based on assumption of ADC's of 0.85 for gross energy and 0.9 for crude protein

Live Weight (g)	<100	100-500	500-1000
DE content (MJ kg^{-1})	17	18	19
DP content (g kg^{-1})	510	468	437
DP:DE (g DP MJ^{-1} DE)	30	26	23
GE	20	21.2	22.2
CP	57	52	49

Table 5.2. Theoretical formulation (% dry matter basis) of soy protein diets to achieve DP:DE requirement of grouper growing from 10 – 1000 g using two different quality fishmeal's; 65% crude protein or 55% crude protein

	Fishmeal (55% CP) Diets			Fishmeal (65% CP) Diets		
Fish Size (g)	**<100**	**100-500**	**500-1000**	**<100**	**100-500**	**500-1000**
Diet	**1**	**2**	**3**	**1**	**2**	**3**
Blood meal	5.55	6.87	7.66	1.74	6.84	9.46
Brewer's Yeast	2.00	8.00	8.00	2.12	8.33	4.00
Choline Chloride (70%)	0.40	0.40	0.40	0.40	0.40	0.40
Fish Oil	2.00	6.00	11.15	2.00	5.70	11.75
FM (65%)	0.00	0.00	0.00	50.00	22.00	22.21
FM (55%)	50.38	23.08	18.74	0.00	0.00	0.00
Krill Meal	0.00	5.98	5.29	0.00	5.00	0.69
Starch	5.00	7.43	7.15	7.70	9.00	13.59
Methionine	0.26	0.44	0.50	0.22	0.44	0.50
Stay-C	0.30	0.30	0.30	0.30	0.30	0.30
SBM	12.11	15.00	15.00	16.00	15.00	16.00
SPC	16.00	15.00	14.00	15.59	16.00	14.41
Soy Lecithin	1.40	1.10	0.00	2.04	2.00	0.34
Taurine	0.93	0.95	0.97	0.89	0.94	0.96
Vit-min premix	0.50	0.50	0.50	0.50	0.50	0.50
Wheat Flour	0.00	3.46	4.00	0.00	4.09	0.00
Wheat Gluten	2.67	5.00	5.85	0.00	2.96	4.37
NaH2PO4	0.50	0.50	0.50	0.50	0.50	0.50

Table 5.3. Theoretical proximate and amino acid composition (dry matter basis) of soy protein diets presented in Table 5.1 and formulated to achieve the requirements for DP:DE for grouper growing from approximately 10 – 1000 g using two different quality fishmeal sources

	55% Crude Protein Fishmeal Diets			65% Crude Protein Fishmeal Diets		
Fish Size (g)	**<100**	**100-500**	**500-1000**	**<100**	**100-500**	**500-1000**
Diet	**1**	**2**	**3**	**1**	**2**	**3**
Dry Matter	92.33	92.27	92.58	91.60	92.00	92.21
Ash	16.38	10.48	9.09	14.16	9.87	8.32
GE (MJ/KG)	20.00	21.20	22.20	20.00	21.20	22.20
Protein	56.00	52.00	50.00	56.00	52.00	50.00
Fat	10.00	12.00	15.40	10.00	12.00	15.40
NFE	17.62	25.52	25.52	19.84	26.13	26.28
Arginine	3.56	3.09	2.90	3.58	3.13	2.90
Cysteine	0.50	0.51	0.50	0.58	0.50	0.50
Glycine	3.40	2.59	2.40	3.28	2.57	2.41
Histidine	1.90	1.58	1.52	1.71	1.55	1.60
Isoleucine	2.09	1.85	1.73	2.14	1.87	1.63
Leucine	4.27	3.98	3.86	4.15	4.08	3.97
Lysine	3.83	3.33	3.15	3.84	3.47	3.27
Methionine	1.40	1.40	1.40	1.40	1.40	1.40
Phenylalanine	2.61	2.53	2.46	2.47	2.47	2.46
Serine	2.38	2.40	2.33	2.66	2.46	2.45
Taurine	1.00	1.00	1.00	1.00	1.00	1.00
Threonine	2.12	2.03	1.95	2.41	2.19	2.09
Tyrosine	1.71	1.67	1.60	1.93	1.75	1.65
Valine	2.85	2.58	2.50	2.50	2.50	2.50

Table 5.4. Proximate and amino acid composition of raw ingredients used to formulate hypothetical diets in Table 5.1. Data expressed on dry matter basis. All raw ingredient nutrient profiles from PSFI laboratory data as analyzed with the exception of Fishmeal (55%) where data was adapted from Zaviezo and Dale (1994). NA: Not Available

Composition (%)	Blood Meal	Brewer's Yeast	Fishmeal (65%)	Fishmeal (55%)	Krill Meal	SBM	SPC	Starch	Wheat Flour
Dry Matter	92	97	93.14	94.3	93.4	87.6	91.6	86.78	87.59
Ash	1.8	5.6	21.44	26.19	12.3	6.8	6.2	0.52	0.56
GE (MJ/KG)	24.3	19.9	19.68	19.12	23.6	19.5	20.3	17.1	18.1
Protein	97.8	47.9	64.38	55.78	61.6	54	72.5	0	12.88
Fat	0.5	2.8	10.46	11.77	20.2	2.6	1.2	0.33	1.428
NFE	-0.1	43.7	3.7	6.26	5.9	36.6	20.1	99.2	85.1
Alanine	7.1	2.8	4.04	NA	3.2	2.3	3.1	0	0.32
Arginine	4	2.4	4.01	3.76	3.7	3.9	5.3	0	0.41
Aspartic acid	9.1	4.7	6.03	NA	6.4	6.3	8.4	0	0.44
Cysteine	0	0.5	0.62	0.4	0.5	0.8	0.9	0	0
Glutamic acid	8.1	7.5	8.74	NA	8	10.3	13.7	0	4.09
Glycine	3.9	2.1	4.66	4.61	2.8	2.2	3	0	0.4
Histidine	5.6	1	2.17	2.14	1.2	1.4	1.8	0	0.31
Isoleucine	0.8	2	2.56	2.38	2.9	2.1	3	0	0.43
Leucine	11.6	3.1	4.8	4.11	4.7	3.9	5.5	0	0.78
Lysine	8.7	3.5	4.78	4.24	4.2	3.2	4.5	0	0.24
Methionine	1.5	0.7	1.71	1.48	1.8	0.8	1	0	0.18
Phenylalanine	6.7	2	2.62	2.34	3	2.7	3.6	0	0.56
Proline	3.6	2.2	4.13	NA	3.2	3.1	4.8	0	1.34
Serine	5.1	2.4	2.85	1.89	2.7	3	4	0	0.58
Taurine	0	0	0.28	0.2	0.5	0	0	0	0
Threonine	4.9	2.3	2.96	2.01	2.9	2.1	2.9	0	0.31
Tyrosine	2.9	1.6	2.3	1.63	2.7	2	2.4	0	0.27
Valine	8.7	2.3	2.97	2.99	2.9	2.2	3	0	0.51

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Dr. Pirozzi is an Aquaculture Nutritionist and has worked on several key marine fish species of commercial importance including yellowtail kingfish, barramundi, mulloway and grouper, as well as invertebrate species including abalone, prawns, sea urchin and crayfish.

He has 20 years' experience in Aquaculture Research. His research interests and expertise are in sustainable feeds and diet development, protein and amino acid nutrition, bioenergetics, fish physiology and metabolism. He is an aquaculture nutrition consultant and trainer and has provided services for a number of Australian and International organisations. He has worked on a long-term project with the US soy industry to better understand the nutritional requirements of different grouper species which has led to this summary paper.

Soy In Aquaculture Program

This technical paper was created through the USSEC Soy In Aquaculture (SIA) program and the USSEC Southeast Asian Regional Program. USSEC works with target audiences in Southeast Asia and globally to show the utility and benefits of using United States soybean products in aquaculture diets.

The SIA program replaces the Managed Aquaculture Marketing and Research Program (the AquaSoy Initiative, funded and supported by the United Soybean Board and American Soybean Association) which was designed to remove the barrier to soybean meal use in diets fed to aquaculture species.

The objective of the SIA is to optimize soy product use in aquaculture diets and to create a preference for U.S. soy products in particular, including but not limited to U.S. soybean meal, soybean oil, soybean lecithin, and "advanced soy proteins" such as fermented soy and soybean protein concentrate.

This paper follows the tradition of USSEC to provide useful technical materials to target audiences in the aquaculture industry.

For more information on soybean use in aquaculture and to view additional technical papers, please visit the Soy-In-Aquaculture website at www.soyaqua.org.

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