

AMERICAN UNIVERSITY OF BEIRUT

SUBSTITUTION OF FISH MEAL BY SOYBEAN MEAL IN
DIETS FOR JUVENILE MARBLED SPINEFOOT *SIGANUS*
RIVULATUS.

by
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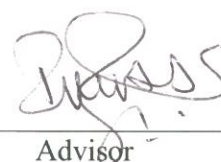
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Title: Substitution of Fish Meal by Soybean Meal in Diets for Juvenile Marbled Spinefoot *Siganus rivulatus*.

The challenge of replacing fish meal protein with alternative plant protein sources in aquafeeds for cultured species is an important aspect of modern aquaculture. The present study was conducted to assess the extent to which fish meal could be replaced with soybean meal as a source of dietary protein for marine herbivore *Siganus rivulatus*. Five iso-nitrogenous (40% CP) and iso-energetic (14MJ/Kg) diets were prepared with SBM replacing fish meal at 0, 25, 50, 75, and 100% levels of dietary protein. An 8-week feeding trial was performed with 240 juvenile *S. rivulatus* (mean weights 1.74 ± 0.03 g) size-sorted and stocked into 15 tanks randomly assigned in triplicates to each of the five treatments. The effect of SBM on survival, growth, condition, feed efficiency, protein efficiency and protein utilization in marbled spinefoot was assessed. A 3-week follow-up feeding trial using fish from the first experiment was performed to assess the effect of SBM in the diets on hematological parameters in *S. rivulatus*. Growth correlated negatively with increase in SBM inclusion, with best fit regression analysis of final body weights (g) resulting in a straight line with $R^2 > 0.91$. Inclusion of SBM at high levels in the diet negatively affected SGR, FE, PER and GPU, with 100% SBM in diet resulting in smallest K and least values in all growth parameters among treatments. No significant effect on VSI, whole body protein and body ash was observed while HSI and body lipid decreased significantly with increased SBM inclusion. There was no observed immediate effect on total or differential blood counts, hemoglobin, or plasma protein, except for a statistically non-significant increase in neutrophils with increase in SBM inclusion. Hematocrit expressed a negative correlation with SBM and decreased significantly when 100% SBM was supplied in the diet. The results of the present study suggest that SBM is unsuitable for use as alternative to fish meal in diets for juvenile marbled spinefoot, and further investigations are necessary to determine the extent of possible partial use of SBM in the diets for this marine herbivore.

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And indeed there will be time

.....

*Time for you and time for me,
And time yet for a hundred indecisions,
And for a hundred visions and revisions,
Before the taking of a toast and tea.*

T.S. Eliot (1888-1965)

*To my dear parents and my family back in the far but never forgotten homeland,
Низкий Вам Поклон!*

CHAPTER I

INTRODUCTION

A. Introduction

Aquaculture is broadly defined as rearing and culture of “aquatic animals and plants in fresh, brackish and marine environments” (Pillay & Kutty 2005). Aquaculture includes for various nutrient-rich foods such as finfish, mollusks, crustaceans, amphibians and a variety of aquatic animals such as jellyfish and sea urchins, as well as various aquatic plants and algae (FAO 2014). In addition to human food production, aquaculture could provide for highly-valued nutrients used in feeds for farmed animals (swine, poultry, fish, etc.). Modern aquaculture is a fast-growing industry contributing amply to global fish production (FAO 2014). In 2012, aquaculture alone contributed to 42% of total fish production, supplying 49.5% of all aquatic animal and plant production for human consumption (FAO 2014).

Global capture fisheries’ production has experienced reduction in growth over the past decade, with annual production currently stable at around 90 million tons per year and majority of fish stocks established as fully fished (FAO 2014). Aquaculture production is, on the contrary, steadily increasing, with 58.3 million people employed in the sector in 2012 and employment rates growing faster than in other agriculture sectors (FAO 2014). As the industry continues to grow, development of more sustainable aquaculture practices is imperative if we are to maintain and increase current production.

Fish meal has been widely used as major protein source for animal and fish feeds for a long time. Fish meal is most often produced from pelagic fish such as

anchovy and menhaden as well as offal from herring and salmon processing (Hardy & Barrows 2002) and is therefore a source of high-quality protein with an excellent amino-acid profile, long-chain omega-3 fatty acids, and various vitamins and minerals necessary for good health and growth of farmed animals (Olsen & Hasan 2012). Unfortunately, wild-caught fish meal is not a sustainable resource especially when most high-value marine fish species farmed are carnivores (such as salmon) feeding at high trophic levels and requiring high protein inclusion in the diet. Terrestrial plants on the other hand constitute a sustainable resource of good-quality protein, and various plant – protein meals (wheat- and corn-gluten, rapeseed/canola, cottonseed, sunflower, soybean, etc.) have been successfully used to supplement and partially substitute fish meal in diets for herbivore/omnivore and high-quality carnivorous species (Dersjant-Li 2002; Kaushik et al. 2004; Gatlin et al. 2007; Lim et al. 2008).

B. Soybean Meal as Alternative to Fish Meal

Soybean (*Glycine* sp.) is a leguminous plant introduced almost everywhere around the world but initially native to Asia (Brown et al. 2008). Soybean seeds are classified by nutritionists as oilseeds because of their relatively high lipid content. Raw soybean seeds are processed and flaked, and full-fat flakes are subjected to lipid extraction (usually with solvent) to produce high-protein (~49% CP) soybean meal (SBM) used for human and animal consumption (Brown et al. 2008). Soybean meal is one of the most commonly used plant-protein sources (FAO 2014) and was used to replace fish meal in the diets of various fish species resulting in a wide range of suitability (Shiau et al. 1988; Lim et al. 2011; Lin & Luo 2011; Ye et al. 2011; Colburn et al. 2012; Silva-Carrillo et al. 2012; Yu et al. 2013). The price of SBM in the market,

even though on the rise since 2006, is generally less than that of fish meal, and has remained relatively stable when compared to ever-rising fish meal prices (Olsen & Hasan 2012).

1. Anti-Nutritional Properties of SBM

SBM has several negative aspects related to its inclusion in feeds. Soybean meal amino-acid profile is deficient in methionine, lysine and threonine (Brown et al. 2008). Soybeans also contain several anti-nutrients or anti-nutritional factors (ANFs). These “antinutrients” can be defined as substances and/or their metabolic products which interfere with food utilization in the animal, consequently affecting its performance (Francis et al. 2001). Anti-nutritional factors found in soybean meal include trypsin inhibitors; lectins (glycoproteins); glucosides known as saponins; phytates; soybean carbohydrates (oligosaccharides and NSPs); as well as several antigenic proteins with allergenic properties, goitrogens, hydrocyanic acid, and urease, among others. (Baeverfjord & Krogdahl 1996; Bakke-McKellep et al. 2000; Dersjant-Li 2002; Krogdahl et al. 2003; Sanden et al. 2005; Brown et al. 2008; Pettersson & Pontoppidan 2013).

Protease inhibitors are plant proteins which form complexes with digestive enzymes in the gastrointestinal tract, thus inhibiting the proteolytic activity of the latter (Cabrera-Orozco et al. 2013). There are two types in soybeans: Kunitz trypsin inhibitor and Bowman-Birk trypsin/chemotrypsin inhibitor (Francis et al. 2001; Brown et al. 2008). These inhibitors inactivate pancreatic trypsin and chemotrypsin enzymes rendering digestion and amino-acid absorption difficult, resulting in growth reduction. Furthermore, they decrease sulfur availability in the organism as the formed enzyme-

inhibitor complexes are excreted and consequently shift utilization of available sulfur amino-acids from tissue synthesis towards more trypsin/chemotrypsin production (Cabrera-Orozco et al. 2013). However, trypsin inhibitors can be at least partially neutralized by careful heat processing to reduce their presence in the product below critical levels (Francis et al. 2001; Brown et al. 2008), and their negative effects on sulfur availability are compensated by supplementation of sulfur amino-acids in the diet (Ai & Xie 2005).

Lectins (also known as phytohaemagglutinins) are glycoproteins, some of which are anti-nutritional and even toxic when consumed (Cabrera-Orozco et al. 2013). Lectins are resistant to proteolytic activity in the intestine and are capable of binding intestinal epithelial cells, inducing damage to the villi and interfering with normal metabolism in the small intestine (Francis et al. 2001). Lectins were reported by several authors to have induced morphological changes in the distal gut of Atlantic salmon (*Salmo salar* L.), along with reduced nutrient uptake and reduced gut enzyme activity (Baeverfjord & Krogdahl 1996; Bakke-McKellep et al. 2000; Francis et al. 2001; Sanden et al. 2005; Brown et al. 2008; Cabrera-Orozco et al. 2013). Lastly, lectins' anti-nutritional effects tend to be more potent when other ANFs are present in the diet (Francis et al. 2001).

Saponins are a group of glycosides widely distributed in green plants and known for their surfactant properties (Cabrera-Orozco et al. 2013). In soy, triterpenoid glycosides in high concentrations can break cell membranes of the intestinal mucosal cells, increasing their permeability and inhibiting active nutrient uptake (Brown et al. 2008; Francis et al. 2001; Cabrera-Orozco et al. 2013).

Phytates (phytic acid and inositol phosphates) are the main form of phosphorus storage in oilseeds, and are known to exert several anti-nutritional effects when consumed (Cabrera-Orozco et al. 2013). Not only do they render phosphorus unavailable to non-ruminants (because of lack of phytase synthesis in these organisms), but they also bind various ions such as Ca^{2+} and Mg^{2+} , as well as iron and zinc cations, making them unavailable to consumers (Francis et al. 2001). Calcium plays an important role in nutrient uptake by the cell (Brown et al. 2008), and thus high phytate in the diet can decrease nutrient absorption. Phytates also bind proteins, forming phytate-protein complexes and limiting dietary protein availability (Francis et al. 2001). These complexes also affect protein structure which in turn negatively affects enzyme activity, inhibiting proteolytic enzymes among others, consequently reducing absorption, function and digestibility of dietary protein (Cabrera-Orozco et al. 2013). Fortunately, some of these effects can be mitigated by dietary phosphorus supplementation and addition of phytase in the diets (Brown et al. 2008).

Oligosaccharides and non-starch polysaccharides (NSPs) found in soybeans can decrease nutrient digestibility in fish by affecting digestive enzymes or binding bile acids (Francis et al. 2001). Soluble NSPs are particularly dangerous as they obstruct movement of substrates and decrease their digestibility in the intestine by trapping water and forming gum-like masses, increasing viscosity of intestinal contents (Francis et al. 2001).

Soybean meal is known to induce enteritis and alter gut morphology when included in the diets for Atlantic salmon (*Salmo salar*) thus negatively affecting fish growth and feed digestibility (Baeverfjord & Kroghdahl 1996; Bakke-McKellep et al. 2000; Merrifield et al. 2011). Soy saponins are capable of altering the membrane

permeability of the intestinal brush border cells thus disrupting the intestinal barrier and promoting infection (Brown et al. 2008; Merrifield et al. 2011). This process is accompanied by increase in white blood cell (WBC) counts specifically lymphocytes, neutrophils, monocytic lineage cells (macrophages and eosinophils), and mixed populations of putative cells (Baeverfjord & Krogdahl 1996; Bakke-McKellep et al. 2000). However, the exact cause of these inflammatory responses is not attributed to saponins alone but rather several alcohol-soluble components of SBM (Olli & Krogdahl 1995). Because blood composition can change dramatically with stress, malnutrition and pathology, hematology can prove useful in evaluating the effects of dietary SBM on fish guts (Vosylieniė & Kazlauskieniė 1999).

C. Marbled Spinefoot *Siganus rivulatus*

1. Species' Biology

Marbled spinefoot *Siganus rivulatus* (Forsskål 1775) is a member of the Siganidae family of algae-eating tropical Indo-Pacific fishes (Woodland 1983). There are 27 rabbitfish species and two genera, *Siganus* and *Lo*, the former being more abundant. Most rabbitfish species have a drab coloration and are gregarious in nature, and only a few are territorial (Duray 1998). The schooling species are of interest to aquaculturists for obvious reasons. *S. rivulatus* is a Lessepsian invader that migrated into the Mediterranean from the Red Sea through the Suez Canal after its opening in 1869 (Ben-Tuvia et al. 1972; Bariche 2005). It was first detected by Steinitz in 1927, and had since established considerable populations in the Mediterranean (Duray 1998; Bariche 2005). *S. rivulatus* has an ovoid, laterally compressed body shape, small head, and a rounded snout which gives it resemblance to a rabbit (Duray 1998). It has

numerous sharp teeth which help it in grazing, and two sets of venomous spines: 13 on the dorsal and 7 on the ventral sides (Anastasiades 2011). Marbled spinefoot are coastal dwellers, appearing in big schools in coastal shallow areas. In nature, marbled rabbitfish are herbivores feeding on various macrophytes in a selective fashion (Bariche 2006), reaching a maximum size and weight of 32cm and 318g respectively (Bariche 2005; Anastasiades 2011). The spawning in the fish occurs between May and August and is synchronised with the lunar cycle (Duray 1998; Anastasiades 2011).

2. Culture Potential

There is special interest in culture of *S. rivulatus* as it is a marine herbivore. Decreasing freshwater availability and pressure on fisheries resources favor production shift towards marine herbivorous fishes (Naylor et al. 2000). Herbivores are considered to have better energy efficiency than carnivorous fish, lower energy need in their diet (Anastasiades 2011), and better utilization of plant-based diets (Bowyer et al. 2013b) which in effect implies less fish meal and fish-oil use in feeds. Despite being primarily herbivorous in nature, *S. rivulatus* was found to be omnivorous in captivity and readily accepts artificial feed (Ben-Tuvia et al. 1972; Lam 1974; Boonyaratpalin 1997; El-Dakar et al. 2011). Its invasive history in the Mediterranean is suggestive of the species' sturdiness and tolerance to physiological stress as the Suez Canal is a serious invasion barrier characterized by shallowness, and temperature and salinity extremes (Galil 2000). Indeed, marbled spinefoot was reported to be able to tolerate high densities in culture (Ben-Tuvia et al. 1972; Saoud et al. 2008b), and is a euryhaline and eurythermal species capable of withstanding wide salinity (10 – 50 ppt) (Saoud et al. 2007) and temperature (17 – 32°C) ranges (Saoud et al. 2008c). In culture, marbled spinefoot

optimum growth temperature and salinity were reported by Saoud et al. (2007, 2008c) to be 27°C and 35 ppt respectively. Marbled spinefoot was successfully spawned in captivity by researchers in Cyprus (Stephanou & Georgiou 2000) and F₃ generations have been reared successfully in controlled environments. Finally, the fish has a good reputation in the local Levantine market (Cyprus, Saudi Arabia, Lebanon, ...) (Anastasiades 2011) because of its good flesh quality and tastiness (Saoud et al. 2008a; Anastasiades 2011), and is cultured commercially in sea cages in Cyprus, and experimentally in Saudi Arabia (Lichatowich et al. 1984; Bukhari 2005; Anastasiades 2011).

D. Aim of Present Study

Sustainability research in finfish nutrition has been ongoing for a long time and has successfully reduced fish meal use in aquafeeds through substitution with alternative protein sources. Furthermore, farming of organisms feeding low on the food chain should allow for easier inclusion of plant protein in the diet, without considerable effect on the market size and quality of the end product. In the present study, we assessed optimal inclusion levels of solvent extruded soybean meal as an alternative plant-based source of protein in diet of marbled spinefoot *Siganus rivulatus*. We also studied the effects of dietary SBM on hematological parameters of the fish.

CHAPTER II

MATERIALS AND METHODS

A. Experimental Diets

Five iso-nitrogenous and iso-energetic (14 MJ/Kg) diets were formulated (**Table 2.1**). The diets contained 40% crude protein and 5.4% crude lipid. SBM as dietary protein source was supplied in diets at 25% (D2), 50% (D3), 75% (D4), and 100% (D5) replacement of FM. Digestible energy values were calculated as 33.5, 16.7, and 16.7 KJ/g for lipid, protein, and nitrogen-free extract respectively (Bureau et al. 2002). Cod liver oil was used as source of lipids. Digestible energy of the prepared diets was maintained by adjusting the levels of wheat flour and cellulose. Methionine in crystalline form was supplemented to the diet as this amino-acid is limiting in soy (Brown et al. 2008). Ingredients were mixed with hot water using a dough mixer and extruded in a meat grinder (3.5mm die). Gelatin was used as binder for the pellets. Pellets were dried in a forced-air oven at 40°C to a moisture content of 8%, ground to suitable size, and stored in airtight zip-lock bags at -20°C. Chemical composition of diets was determined by proximate analysis. Protein, lipid, and ash proportion (% dry matter) in the five diets were approximately 42%, 5%, and 11% respectively.

Table 2.1: Ingredients and chemical composition of the five diets with increasing inclusion of SBM to replace protein supplied by fishmeal in diets offered to juvenile *S. rivulatus* over 8 weeks.

Diet	D1	D2	D3	D4	D5
SBM as % of dietary protein	0	25	50	75	100
Menhaden Fishmeal ¹ (g/Kg diet)	521.5	392.0	268.0	133.1	0.00
Soybean meal solvent extracted ² (g/Kg diet)	0.00	197.0	384.0	588.0	790.0
Cod Liver Oil ³ (g/Kg diet)	0.00	11.9	23.8	35.6	47.3
Wheat flour (g/Kg diet)	340.0	264.0	187.5	112.0	38.0
Vitamin & Mineral premix ⁴ (g/Kg diet)	30.0	30.0	30.0	30.0	30.0
Choline chloride (g/Kg diet)	5.0	5.0	5.0	5.0	5.0
Stay C 250 ⁵ (g/Kg diet)	1.0	1.0	1.0	1.0	1.0
CaP-dibasic (g/Kg diet)	2.0	16.0	31.0	45.0	60.0
Cellufill ⁶ (g/Kg diet)	88.5	70.1	55.8	35.4	12.8
DL-Methionine (g/Kg diet)	2.0	3.0	3.9	4.9	5.9
Gelatin ⁷ (g/Kg diet)	10.0	10.0	10.0	10.0	10.0
Chemical Composition (g/100g in dry matter)					
Crude Protein [†]	38.62	38.66	38.61	38.59	38.60
Crude Lipid [†]	5.22	5.29	5.41	5.42	5.44
Digestible Energy [†] (MJ/Kg)	13.94	14.06	14.1	14.24	14.41
Phosphorus [†]	1.67	1.72	1.81	1.85	1.92

¹ FF Skagen Denmark. Havnevagtvej 12.9990 Skagen.

² De-hulled solvent extracted soybean meal, Southern Sates Cooperative Inc., Richmond VA, USA.

³ Seven Seas LTD., Great Britain. Mar Fleet, Hull, England HU9 5NJ.

⁴ The vitamin and mineral premix provided the following per kg of experimental diet: vitamin A retinyl acetate 1 million IU, vitamin D3 cholecalciferol 0.1 million IU, vitamin E alpha-tocoph acet 7 g, vitamin K 0.5 g, folic acid niacin 0.1 g, niacin 4 g, calcium pantothenate 2.5 g, riboflavin (B2) 0.6 g, vitamin B12 0.001 g, thiamine (B1 nitrate) 0.5 g, pyridoxine (B6 HCl) 0.5 g, biotin 0.0125 g, vitamin C (ascorbic acid) 0.25 g, inositol 5 g, selenium (as sodium selenite) 0.0045 g, iodine (as calcium iodate) 0.25 g, iron (as sulphate monohydrate) 2 g, zinc (as oxide) 5 g, copper (as sulphate pentahydrate) 0.25 g, manganese (as sulphate monohydrate) 3.5 g, chlorine chloride 75, phosphorus (as monocalcium phosphate) 2.5, sodium chloride (salt) 225 g, and cellulose 75 g. Calcium carbonate carrier to balance.

⁵ 250 mg kg⁻¹ active vit C supplied by Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), Roche Vitamins Inc., Parsippany, New Jersey, USA.

⁶ Alpha-cellulose, Unites States Biochemical Corporation, Cleveland, Ohio, USA.

⁷ Himedia laboratories Pvt. Ltd., 23, Vadhani Ind. Est., LBS Marg, Mumbai, India.

[†] Based on a calculated value.

B. Experiment 1: Growth

1. Fish Acquisition and Experimental Design

Juvenile marbled rabbitfish were caught off the northern shore of Ras-Beirut and transported to the aquaculture research facility at the American University of Beirut (AUB). Fish were quarantined in an outdoors recirculating system for one month and trained to accept commercial feed (36% Crude Protein, 6% Crude Fat; Rangen EXTR 350, Rangen Inc., Buhl, Idaho) twice a day. At trial start, fish were hand-sorted to a uniform size and 30 fish taken at random and used to calculate initial Fulton's condition indices ($K = 10^5 \times W/L^3$ where W is weight (g) and L is length (mm)). Fish were then stocked into an indoors recirculating system consisting of 15 fiberglass tanks (65×64×55cm; L×W×H) connected to a bio-filter and sump tank (the tanks were filled only to three quarters of holding capacity during the trial i.e. approximately 172 L). Aeration was provided using a regenerative blower and submerged air-diffusers. Photoperiod was maintained at 14:10 hrs (light:dark) with the light phase starting at 6:00 and ending at 20:00. Twenty one fish at the start of the feeding trial were frozen whole at -20°C, then divided into four groups and stored as initial sample for proximate analysis.

Dissolved oxygen (DO) concentration, salinity, and water temperature were measured using a YSI 85 (Yellow Springs Inc., Ohio, USA) and recorded daily. Water temperature was maintained at 26.4 ± 0.4 °C (mean \pm SE) using submersible heating elements. Salinity was maintained at 35.7 ± 0.05 ppt (mean \pm SE). Dissolved oxygen concentration (DO) remained greater than 5 mg/L (6.1 ± 0.04 mg/L). Water quality parameters (pH, Total Ammonia Nitrogen (NH₃-N) and Nitrite nitrogen (NO₂-N)) were measured weekly using a HACH Saltwater Aquaculture Test Kit, Model FF-3, and

averaged 7.8 ± 0.05 (mean \pm SE), 0.06 ± 0.00 mg/L NH₃-N and 0.06 ± 0.02 mg/L NO₂-N, respectively.

Sixteen fish (1.11 ± 0.003 g; mean \pm SE) were stocked into each of the 15 tanks. A set of three tanks was assigned one of the five treatments at random, resulting in three replicate tanks per treatment. Fish were offered experimental diets four times daily (7:00, 11:00, 15:00, and 19:00), and ration calculated as 6% of the largest fish biomass divided into four equal feeding events. Fish were offered feed manually and feed consumption confirmed by observation. All tanks were routinely monitored for leftover feed to be removed if not consumed within a period of half-hour from feed delivery into the tank. Any dead fish were immediately removed from the tank and recorded. Fish were group weighed biweekly after a day of fasting and ration adjusted accordingly. Ration was increased by 10% on weeks when biomass was not measured. The first two weeks after stocking were considered an acclimation period for the fish and any mortalities were replaced with equal-sized fish. First weighing was two weeks after stocking. Individual weights were 1.74 ± 0.03 g (mean \pm SE). No mortality replacements were made here forth and daily ration was adjusted with changes in fish number per tank. The feeding trial was terminated after 60 days.

2. Sampling and Chemical Analysis

At termination, all fish were anaesthetized with a solution of Tricaine-S (Tricaine Methanesulfonate, MS 222, Western Chemical Inc., Ferndale, WA, USA), group weighed, their individual weight and length recorded, and final condition indices calculated. Four fish from each tank were taken at random and dissected, viscera and liver weighed and hepatosomatic (HSI) and viscerosomatic (VSI) estimated. Samples

from each tank were then pooled and macerated whole (along with dissected organs) in a blender. Pooled samples were dried in an oven at 95°C until constant weight and body moisture determined. Dried samples were ground using a mortar and pestle and stored at -20°C for further analysis.

Lipid content was determined using a Reflux extractor (ANKOM XT20 Fat Analyzer and ANKOM^{XT} Recovery System, ANKOM Technology Corporation, Macedon, NY, USA) and petroleum ether as solvent. Lipid content in the initial samples was determined by Soxhlet method because the sample size was insufficient for analysis with Reflux extractor. The samples were weighed into prepared ANKOM extraction bags which were then folded and fit into ceramic thimbles in groups of two. The solvent used was a mixture of petroleum ether/diethyl ether (9:1). Protein content in the samples was determined by a modified Kjeldahl method using Digesdahl Digestion Apparatus with sulfuric acid (H₂SO₄) and hydrogen peroxide (H₂O₂) as oxidizing solutions (Brayton 1992). A 0.1 gm pre-dried sample was digested in 4 ml of concentrated sulfuric acid (H₂SO₄) at 440°C, in the presence of 10 ml of 50% hydrogen peroxide (H₂O₂). The obtained digest was then diluted with de-mineralized water and treated with Nessler reagent for spectrophotometric analysis. Absorbance of the sample at 460 nm was then read using HACH spectrophotometer (Drell 2400) and value reported in mg/l TKN (Total Kjeldahl Nitrogen). Percent (%) nitrogen was calculated using the given formula for solid sample analysis: $((A \times 75) / (B \times C)) / 10000$ where (A) is the spectrophotometer reading obtained in mg/l TKN, (B) is the sample amount used (0.1g), and (C) is the sample volume of digest used (0.5 ml). Nitrogen values were multiplied by a correction factor (1.0739) determined using glycine as a standard (5.665% N, 35.40% CP). Protein content of samples was estimated by multiplying the obtained

values by 6.25 (Albanese & Orto 1963). Ash content was determined by combustion of 0.5 g of dried sample at 500°C for 8 hours. All proximate analysis results were reported on a wet weight basis.

C. Experiment 2: Hematology

1. Experimental Design

Twelve fish were taken from each treatment in previous experiment and stocked into 10 tanks, each two tanks assigned to the corresponding treatment and diets offered to fish in each tank daily for an additional period of 3 weeks (22 days) to assess effects of SBM on hematology. This trial was deliberately delayed to minimize any handling-related effects on fish blood parameters and allow the fish to grow a bit more. The recirculating system utilized was the same one used for the growth experiment, and experimental conditions of the first experiment were maintained.

2. Sampling and Chemical Analysis

At termination, groups of four fish from each tank were taken at random for blood sampling. Fish were anaesthetized using a solution of Tricaine-S (Tricaine Methanesulfonate, MS 222, Western Chemical Inc., Ferndale, WA, USA). Blood samples (0.5 – 1 ml) were collected by cardiac puncture using a 1 ml syringe with 27 gauge, 2.5 cm heparinized needles and transferred into heparinized 3ml eppendorf tubes. Hematological and biochemical parameters were assessed as described by Nasser (2012), and all procedures performed within 24 hours of sample collection.

Total RBC and WBC counts ($\times 10^6$ cells/ μ l and cells/ mm^3 , respectively) were determined using a modified Neubauer hemocytometer where 20- μ L blood sample was mixed with 4 ml's of Natt-Herrick stain. Differential WBC counts were obtained from

blood smears. The blood smears were prepared within 4 hours of sample collection, fixed with methanol and stained with the Wright-Giemsa stain for leucocyte determination (adopted from Nasser 2012). A total of 800 white blood cells per slide were counted and each cell identified and recorded according to Ainsworth (1992) and Ellis (1976). Each type of blood cell was expressed as percentage of the total number of cells counted.

Hemoglobin (HGB) (mg/dL) was determined by cyanmethemoglobin technique using Drabkin's reagent (Drabkin & Austin 1935). Hematocrit (PCV) was determined by drawing blood into heparinized microhaematocrit tubes followed by centrifugation at 10,000 G for 5 minutes in microhematocrit centrifuge at room temperature (Morris & Davey 2001). PCV was expressed as a percentage (%) of the volume using a micro-hematocrit reader (Nasser 2012). Total plasma protein (g/dl) was measured from the plasma obtained using a veterinary refractometer (RHC-200ATC, Westover Scientific, Inc., Mill Creek, WA, USA) after centrifugation at 10,000 G for 5 minutes (Nasser 2012).

D. Data Analysis and Statistics

Survival was calculated as $(S \%) = (\text{number of fish at the end of experiment} / \text{number of fish at the start of the experiment}) \times 100$. Specific growth rate was calculated as $SGR (\% \text{ day}^{-1}) = (\ln \text{FBW} - \ln \text{IBW})t^{-1} \times 100$ where FBW and IBW are final and initial body weight in grams, respectively, and t is time in days (Hopkins 1992); Feed Efficiency was calculated as $FE = [\text{weight gain of fish (g)}] / [\text{weight of feed offered (g)}]$; Protein efficiency ratio was calculated as $PER = [\text{weight gain (g)}] / [\text{protein offered (g)}]$; Gross Protein Utilization was calculated as $GPU = ([\text{Final protein content in fish$

(g) - Initial protein content in fish (g)] / total amount of protein offered (g));

Hepatosomatic (HSI) and viscerosomatic (VSI) indices were calculated as $HSI = (\text{liver weight (g)} / \text{body weight (g)}) \times 100$ and $VSI = (\text{viscera weight (g)} / \text{body weight (g)}) \times 100$.

All data were expressed as mean values of the three replicates and compared using one-way ANOVA. Significant differences among treatment means were analyzed using Student Newman-Keuls multiple-range test. Differences were considered significant at $P < 0.05$. Statistical analysis of the data was performed using SPSS statistics software (V.12 for Windows, SPSS Inc., USA).

CHAPTER III

RESULTS

A. Experiment 1: Growth

Survival, total length, initial and final body weights, specific growth rate, and condition indices of fish are presented in **Table 3.1**. Growth curves of fish in the five treatments are presented in **Figure 3.1**. Survival did not vary significantly among treatments but was greatest (95.7 ± 2.16 %; mean \pm SE) in fish offered D1 and least (82.2 ± 5.30 %) in fish offered D5. Survival seemed to decrease with an increase in dietary SBM inclusion.

At the end of the feeding trial, final body weights of fish were significantly different among treatments. Average weight of fish in treatment D1 (17.23 ± 0.49 g) was significantly greater than weight of fish in treatments D3, D4, and D5 but not significantly different from that of fish in treatment D2 (16.27 ± 0.35 g). Weight of fish offered D2 was not different from weight of fish offered D3 (**Table 3.1**). Fish offered D4 and D5 had significantly lesser body weights compared to other treatments but weights did not differ significantly between the two treatments. Treatment D5 yielded lowest average body weight among treatments (12.55 ± 0.45 g). Best fit regressions were performed for all data and the results fit a straight line model with equation $y = -0.0058x + 17.33$ and $R^2 = 0.9116$ (**Fig 3.2a**). Breakpoint analysis of final body weights (g) was performed for treatments D1, D2, and D3 and treatments D4 and D5. The results fit straight line models with equations $y = -0.0052x + 17.227$ ($R^2 = 0.7362$) (D1, D2, D3) and $y = -0.007x + 18.068$ ($R^2=0.71$) (D4, D5) respectively, and the projected

maximum level of dietary SBM replacement of FM determined to be at 467.2 g per kg of diet (**Fig. 3.2b**). In general, fish grew slower as SBM inclusion in the diet increased and FM decreased.

Total length at harvest was not different in fish offered D1 or D2 (10.87 ± 0.15 cm and 10.8 ± 0.10 cm, respectively) but was significantly greater than length of fish offered D4 and D5 (10.2 ± 0.15 cm and 10.1 ± 0.20 cm). Total length of fish offered D3 (10.53 ± 0.03 cm) was not significantly different from length of fish in all treatments.

Fulton's condition index (K) did not differ significantly among treatments D1, D2, D3 and D4. K in treatment D5 was significantly less than in other treatments. Specific growth rate (SGR) was significantly greater in fish offered D1 (3.85 ± 0.01 % day⁻¹) than in fish in other treatments but not significantly different among fish offered Diets 2, 3, and 4. SGR was least in fish offered D5 (3.36 ± 0.05 % day⁻¹). Specific growth rate tended to decrease with an increase in SBM inclusion in the diet.

Feed efficiency followed a trend similar to SGR, with D1 yielding the best FE value (0.8 ± 0.02) among all treatments (**Table 3.1**). Feed efficiencies in D2 and D3 were not significantly different from each other. D4 and D5 resulted in significantly lesser feed efficiency compared to other treatments with D5 yielding the least FE value (0.56 ± 0.02). Protein efficiency ratio (PER) was significantly greater in fish offered D1 (1.87 ± 0.05) and D2 (1.83 ± 0.04) when compared with fish in other treatments but not significantly different between the two treatments. Using D5 resulted the smallest PER among treatments (1.29 ± 0.04) (**Table 3.1**). Gross protein utilization (GPU) followed the same trend as PER, with the greatest gross protein utilization found in fish offered D1 and D2 (0.32 ± 0.01 and 0.33 ± 0.005 , respectively) and the smallest GPU observed

in fish offered D5 (0.22 ± 0.002). Both GPU and PER tended to decrease as dietary SBM inclusion increased.

HSI and VSI values are presented in **Table 3.2**. Hepatosomatic indices (HSI) of fish were significantly different from each other among treatments. Fish offered D1 had significantly greater HSI (2.57 ± 0.13 %) than fish in all other treatments. The general trend exhibited was a marked decrease in HSI of fish as SBM inclusion increased. Viscerosomatic index (VSI) on the other hand did not show any significant differences among the five treatments.

Whole body proximate compositions as a proportion of wet weight of fish are presented in **Table 3.3**. Moisture proportion was significantly greater in the fish at stocking (75.30 ± 0.39 %) than in all fish at termination of the experiment. Fish offered D1 had significantly less moisture (69.11 ± 0.37 %) as compared to fish offered D3 and D5 but not when compared to fish offered D2 and D4. Lipid content was inversely proportional to moisture; wild fish had a smaller lipid proportion compared to aquacultured fish. Whole body lipid proportion tended to decrease with increased dietary SBM inclusion. Protein proportion in aquacultured fish was not significantly different among treatments. However, wild fish had a significantly lesser body protein proportion than aquacultured fish. There were no significant differences in body ash proportion among fish in the various treatments. Wild fish had significantly more ash when compared to fish offered experimental diets.

B. Experiment 2: Hematology

Total and differential blood counts are presented in **Table 3.4**. There were no significant differences in total red blood cell (RBC) counts ($\times 10^6$ cells/ μ L) among

treatments. RBC counts of fish in D3 had the widest range of distribution ($2.41 - 4.31 \times 10^6$ cells/ μL) compared to other treatments. Total white blood cell (WBC) counts (cells/ mm^3) also did not show any significant differences among treatments. Differential blood counts (%) did not differ significantly among treatments. There were no significant differences among treatments in proportions of thrombocytes, lymphocytes, monocytes and neutrophils. Basophils and eosinophils were absent from the counts in all treatments.

Hematocrit (PCV), hemoglobin (HGB), and total plasma protein (TPP) values are presented in **Table 3.5**. Hematocrit (%) was significantly less in fish offered D5 (30.8 ± 0.85 %) as compared to fish offered D1 and D2 (40.0 ± 2.45 % and 41.71 ± 3.34 %, respectively) but not significantly different from that of fish offered D3 or D4. Hematocrit did not vary significantly in fish when compared among treatments D1, D2, D3, and D4 but exhibited a decreasing trend with increase in dietary SBM inclusion. Hemoglobin levels (mg/dL) in fish did not differ significantly among the five treatments. There were no significant differences in total plasma protein (g/dL) levels among fish in all treatments.

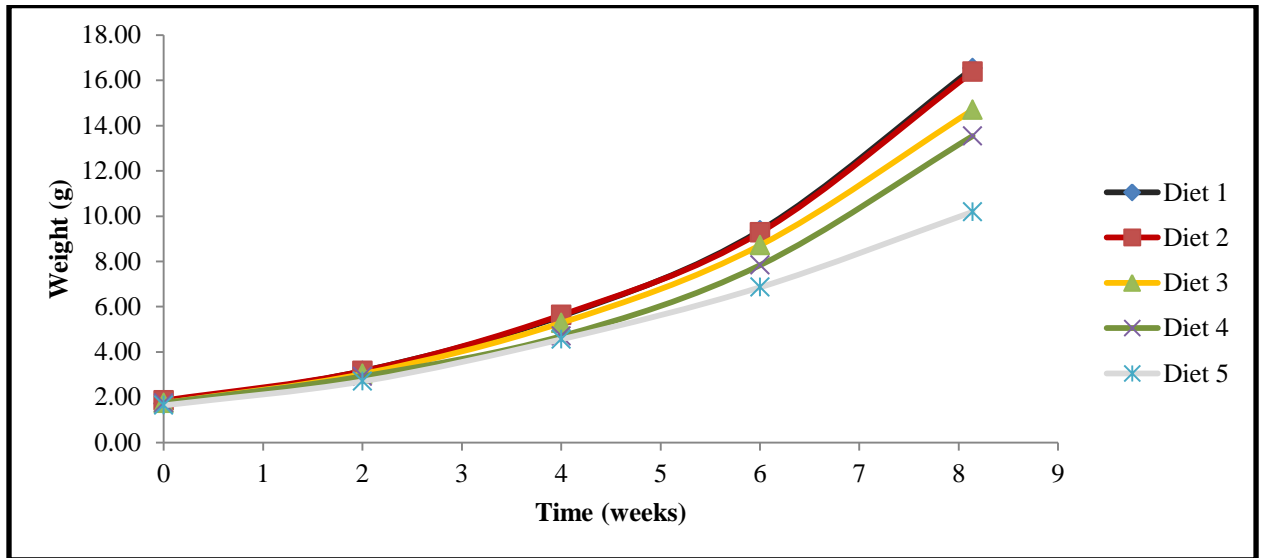


Figure 3.1: Growth in average individual body weight (g) over 8 weeks of juvenile *S. rivulatus*, offered diets with increasing inclusion of SBM.

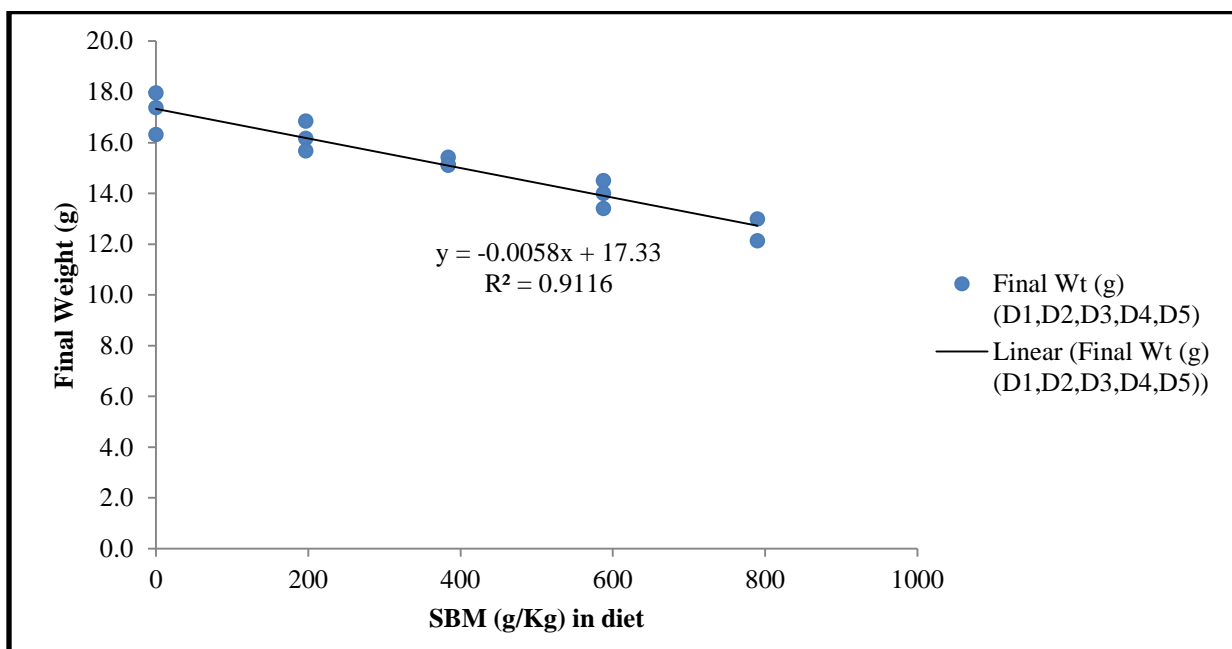


Fig. 3.2a: Linear regression analysis of final body weights (g) as a function of increasing SBM inclusion in the diet (g/kg).

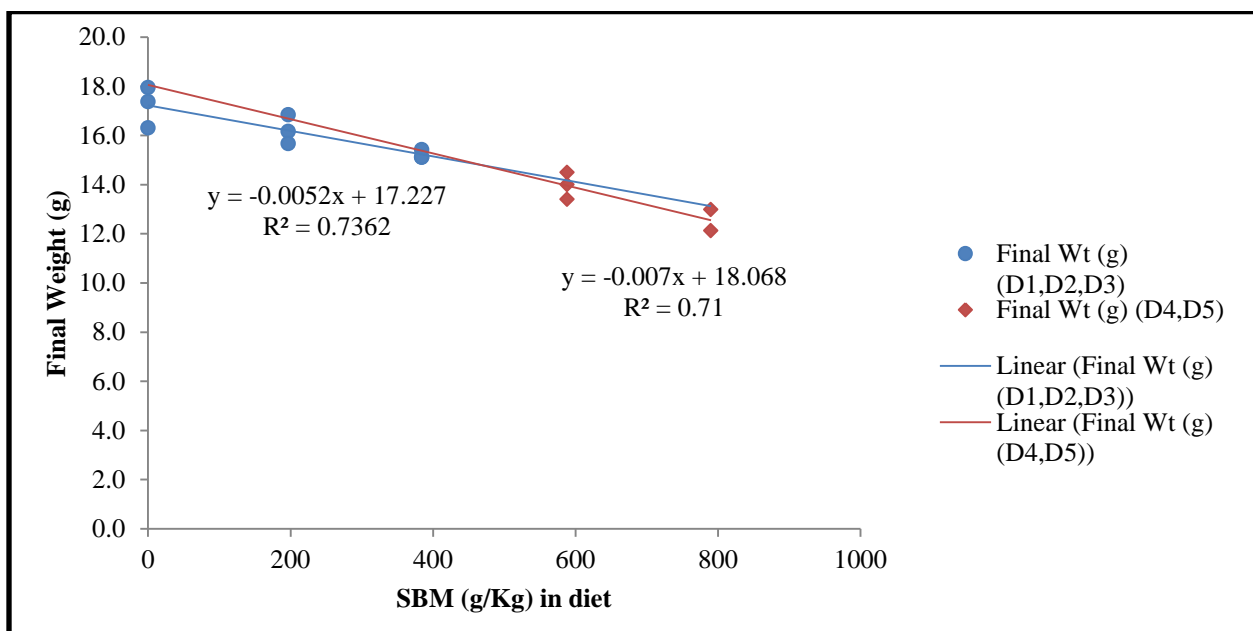


Fig. 3.2b: Breakpoint analysis of final body weights (g/kg) as a function of increasing SBM inclusion in the diet (g/kg).

Table 3.1: Initial body weight (IBW) (g), final body weight (FBW) (g) and total length (TL) (cm) at harvest, Fulton's condition index (K) ($(g/mm^3) \times 10^5$), specific growth rate (SGR; $\% \text{ day}^{-1}$), feed efficiency (FE), protein efficiency ratio (PER), gross protein utilization (GPU) and survival (S; $\%$) values of juvenile *S. rivulatus* offered diets with increasing inclusion of SBM as percentage of dietary protein. Initial sample values are given as (mean \pm SE). Values with different superscripts in the same column are significantly different based on SNK mean separation test ($P < 0.05$).

Treatment	IBW	FBW	TL	K	SGR	FE	PER	GPU	S
D1	1.7 \pm 0.04	17.2 ^a	10.9 ^a	1.32 ^a	3.85 ^a	0.80 ^a	1.87 ^a	0.32 ^a	95.7
D2	1.9 \pm 0.05	16.3 ^{ab}	10.8 ^a	1.28 ^a	3.62 ^b	0.74 ^b	1.83 ^a	0.33 ^a	83.1
D3	1.8 \pm 0.04	15.2 ^b	10.5 ^{ab}	1.27 ^a	3.61 ^b	0.69 ^b	1.58 ^b	0.28 ^b	84.2
D4	1.8 \pm 0.10	13.9 ^c	10.2 ^b	1.28 ^a	3.47 ^{bc}	0.63 ^c	1.59 ^b	0.28 ^b	83.3
D5	1.7 \pm 0.07	12.6 ^d	10.1 ^b	1.20 ^b	3.36 ^c	0.56 ^d	1.29 ^c	0.22 ^c	82.2
PSE ¹		0.35	0.12	0.014	0.048	0.015	0.038	0.007	3.39

¹PSE: Pooled Standard Error

Table 3.2: Hepatosomatic (HSI) (%) and viscerosomatic (VSI) (%) indices of *S. rivulatus* fed diets with increasing inclusion of SBM as percentage of dietary protein. Values with different superscripts in the same column are significantly different based on SNK mean separation test ($P < 0.05$).

Treatment	HSI	VSI
D1	2.57 ^a	12.41
D2	2.24 ^b	12.72
D3	1.81 ^{cd}	12.33
D4	2.03 ^{bc}	12.30
D5	1.63 ^d	13.66
PSE ¹	0.098	0.440

¹PSE: Pooled Standard Error

Table 3.3: Moisture, protein, lipid and ash content as percent of wet matter (as is) of juvenile *S. rivulatus* offered diets with increasing inclusion of SBM as percentage of dietary protein. Values with different superscripts in the same column are significantly different based on SNK mean separation test ($P < 0.05$).

Treatment	Moisture	Protein	Lipid	Ash
Wild fish	75.30 ^a	14.26 ^b	2.93 ^c	5.54 ^a
D1	69.11 ^c	16.90 ^a	10.30 ^a	3.61 ^b
D2	69.89 ^{bc}	17.47 ^a	9.17 ^{ab}	3.70 ^b
D3	70.69 ^b	17.20 ^a	8.45 ^b	3.90 ^b
D4	69.50 ^{bc}	17.33 ^a	8.86 ^{ab}	3.73 ^b
D5	70.66 ^b	16.84 ^a	8.13 ^b	3.93 ^b
PSE ¹	0.389	0.253	0.399	0.111

¹PSE: Pooled Standard Error

Table 3.4: Total counts of red blood corpuscles (RBC) (10^6 cells/ μ L), total white blood cells (WBC) (cells/ mm^3), differential blood cell counts (%) of thrombocytes, lymphocytes, monocytes and neutrophils in juvenile *S. rivulatus* offered diets with increasing inclusion of SBM as percentage of dietary protein. Values for eosinophils and basophils are not shown as none were observed. Values with different superscripts in the same column are significantly different based on SNK mean separation test ($P < 0.05$).

Treatment	Parameter	RBC	WBC	Thrombocytes	Lymphocytes	Monocytes	Neutrophils
D1	Mean \pm SD	4.46 \pm 0.11	88000 \pm 19103	78.00 \pm 8.57	20.00 \pm 4.55	1.50 \pm 1.29	0.80 \pm 1.30
	Range	4.34 - 4.55	72250 - 109250	70 - 91	16 - 26	0 - 3	0 - 3
D2	Mean \pm SD	3.60 \pm 0.19	94500 \pm 24582	81.67 \pm 6.03	15.67 \pm 6.50	1.67 \pm 0.58	1.00 \pm 0.0
	Range	3.39 - 3.74	70250 - 121000	76 - 88	9 - 22	1 - 2	1
D3	Mean \pm SD	3.40 \pm 1.02	63500 \pm 12619	89.50 \pm 3.11	5.75 \pm 4.80	3.75 \pm 2.22	1.50 \pm 0.58
	Range	2.41 - 4.31	49000 - 72000	85 - 92	1 - 11	1 - 6	1 - 2
D4	Mean \pm SD	3.18 \pm 0.62	70312 \pm 11495	71.60 \pm 17.18	24.00 \pm 14.05	3.60 \pm 3.51	1.67 \pm 0.58
	Range	2.51 - 3.78	54750 - 81000	52 - 95	10 - 38	0 - 9	1 - 2
D5	Mean \pm SD	4.07 \pm 0.38	61083 \pm 7509	81.20 \pm 9.55	16.75 \pm 7.76	1.60 \pm 1.52	2.80 \pm 2.49
	Range	3.84 - 4.51	56500 - 69750	71 - 91	9 - 27	0 - 4	1 - 7
PSE ¹		0.359	9675.949	4.779	4.213	1.281	0.859

¹PSE: Pooled Standard Error

Table 3.5: Hematocrit (packed cell volume PCV) (%), Hemoglobin (HGB) (mg/dl), and total plasma protein (TPP) (g/dl) of juvenile *S. rivulatus* fed diets with increasing inclusion of SBM as percentage of dietary protein. Values with different superscripts in the same row are significantly different based on SNK mean separation test (P<0.05). TPP values are not significantly different from each other.

Treatment	PCV	HGB	TPP
D1	40.00 ^a	11.65	4.80±0.14
D2	41.71 ^a	10.81	4.66±0.23
D3	34.36 ^{ab}	11.69	4.86±0.17
D4	36.64 ^{ab}	10.42	4.82±0.22
D5	30.80 ^b	9.62	4.63±0.27
PSE ¹	2.521	0.963	

¹PSE: Pooled Standard Error

CHAPTER IV

DISCUSSION

A. Growth, Condition, and Protein Utilization

In the present feeding experiment, juvenile *S. rivulatus* were offered diets with SBM replacing fish meal at various inclusion levels. Growth of the fish correlated negatively with SBM inclusion in the diet. The high regression coefficient between SBM inclusion and reduction in fish growth suggest that there is no break-point beyond which SBM can be added to the diets without significantly affecting growth but rather that any SBM inclusion in the diet leads to growth reduction in *S. rivulatus*. Similarly, Wang et al. (2006) fed cuneate drum *Nibea miichthioides* diets with increasing SBM inclusion and observed a linear decrease in weight gain with decrease in dietary FM in the diets. Deleterious effects of SBM on fish survival and growth are well known, and were observed in many species. Various authors report growth reduction in fish when fed diets substituting fish meal with SBM, such as Alam et al. (2011) working with juvenile southern flounder *Paralichthys lethostigma*, Lin & Luo (2011) working with juvenile tilapia, Phumee et al. (2011) working with juvenile sutchi catfish *Pangasianodon hypophthalmus*, Ye et al. (2011) working with juvenile japanese flounder *Paralichthys olivaceus*, Bowyer et al. (2013a) working with juvenile yellowtail kingfish *Seriola lalandi*, and Yu et al. (2013) working with juvenile Chinese sucker *Myxocyprinus asiaticus*. Growth reduction of fish was also observed when dietary SBM was mixed with other plant and/or animal-protein. For example, Kikuchi (1999) mixed cottonseed or blood meal into SBM based diets for japanese flounder

P. olivaceus but did not observe improved growth. Similar results were reported by Kikuchi & Furuta (2009) who mixed blue mussel meat into tiger puffer *Takifugu rubripes* SBM based diets, Masagounder et al. (2012), who added cottonseed/bone meal/porcine meal into bluegill *Lepomis macrochirus* diets and Mohsen & Lovell (1990) who added meat and bone meal and/or blood meal in channel catfish *Ictalurus punctatus* diets.

Condition index (K) decreased significantly when 100% of protein in the diet was supplied by SBM. Generally, performance of marbled spinefoot worsened upon complete substitution of fish meal with soybean meal in the diet. Taking into consideration that fish in treatment D5 had significantly lower average body weight and shorter average body length compared to other treatments, this observation partially corroborates the suggestion that substitution of more than 60% of FM protein with SBM leads to adverse effects on fish performance. Furthermore, SGR and FE, which decreased significantly with increase of SBM in the diet, yielded lowest observed values for fish in treatment D5.

Protein efficiency ratio (PER) in *S. rivulatus* correlated negatively with increase in SBM inclusion. Similar results were reported for other omnivorous/herbivorous species such as sutchi catfish *P. hypophthalmus* (Phumee et al. 2011), channel catfish *I. punctatus* (Mohsen & Lovell 1990), milkfish *Chanos chanos* (Shiau et al. 1988), and tin foil barb *Barbodes altus* (Elangovan & Shim 2000). However, these studies report a possible partial substitution of FM by SBM without adverse effects on fish performance. This does not seem to be the case with *S. rivulatus*. Furthermore, GPU of fish in the present study followed a decreasing trend similar to PER. Fish gained less weight and had less protein content in their bodies while amount

of protein offered increased over time. These results, along with decrease in feed efficiency, suggest decreasing assimilation of protein in fish offered increasing SBM proportion in the diet and indicate that despite its herbivorous nature, *S. rivulatus* does not appear to be able to efficiently digest or assimilate SBM sourced proteins. The negative effects on protein utilization and growth in the present work may be explained by the activity of several anti-nutritional factors, namely trypsin inhibitors, lectins, saponins, phytic acid, and soybean carbohydrates (oligosaccharides and NSPs). Inferior protein utilization, low feed efficiency, and reduction in overall growth reported in fish fed SBM diets are often attributed to ANFs present in soy, an effect well known and shown repeatedly in various studies (Baeverfjord & Krogdahl 1996; Bakke-McKellep et al. 2000; Dersjant-Li 2002; Brown et al. 2008; Lim et al. 2011; Ye et al. 2011; Bowyer et al. 2013a; Yu et al. 2013).

B. Hepatosomatic and Viscerosomatic Indices

Hepatosomatic indices (HSI) of the fish in the present experiment do not follow a specific trend. However, HSI in fish receiving full FM treatment were significantly greater than HSI of fish in SBM treatments, which could possibly be explained by ANFs in soy affecting liver growth. This observation is opposite to what is usually reported for fish offered diets with increasing dietary SBM, where increased hepatic lipid deposition results in higher HSI values (Phumee et al. 2011; Ye et al. 2011; Zhou et al. 2005). Results obtained in the present work are similar to findings of Lim et al. (2011) working with tiger puffer *Takifugu rubripes*. Lim et al. (2011) reported decrease in HSI of fish with increased SBM inclusion in diet, and suggested the reason to be impaired or changed liver histology as effect of SBM inclusion. Also, Robaina et al.

(1995) reported increased levels of hepatocyte vacuolization and disorganization in livers of gilthead seabream *Sparus aurata* when 30% of protein in the diet was from SBM. Furthermore, SBM was reported to affect liver enzymatic activity and protein metabolism, and possibly cause liver damage, in juvenile tilapia (*Oreochromis niloticus* × *O. aureus*) (Lin & Luo 2011). It seems that SBM induced liver damage in *S. rivulatus* and consequently affected liver size in the fish when offered at high inclusion levels in the diet, resulting in decreased HSI values and overall reduction in growth, PER, and GPU among treatments. Such results should in future be corroborated using liver histology of rabbitfish offered SBM in their diets.

There were no significant differences observed in VSI of fish among all treatments, but VSI was slightly greater in fish in treatment D2 when compared to full fish meal treatment and when 50 or 75% of protein was supplied by SBM. VSI also tended to increase when 100% of fish meal was replaced by SBM. Marbled spinefoot stores lipids mostly in viscera and muscle (Ghanawi et al. 2011) unlike other species of fish which use liver for lipid storage. Lack of significant differences in VSI in the present experiment could be because there was no increased lipid deposition in the viscera as effect of SBM inclusion. This could mean that dietary energy consumed was immediately utilized by the fish or that the fish used stored lipid reserves for energy when fed diets with increased SBM. Slightly higher VSI in fish offered 25 and 100% SBM protein when compared to other treatments could be the result of ANF-induced morphological changes in the guts of the fish receiving SBM (Baeverfjord & Krogdahl 1996; Sanden et al. 2005). Another possible explanation would be deficiency in dietary lysine upon SBM inclusion and complete replacement of FM in the diet (Peres & Oliveira-Teles 2008). Taking into consideration that soybean meal amino-acid profile is deficient

in lysine (Brown et al. 2008), and that various ANFs in soy decrease nutrient availability, increasing inclusion of SBM protein in the diet might have prompted conversion of inadequate protein into lipid and its consequent storage in the body (Peres & Oliva-Teles 2008; Phumee et al. 2011). However, the possibility of such protein-to-lipid conversion and storage occurring in the present study is low as body lipid proportion in the fish decreased with increase in dietary SBM.

C. Whole Body Composition

Although no significant differences were observed in total protein and ash proportion in the fish in all treatments, lipid and moisture content differed significantly when SBM was included and lipid proportion decreased with increasing SBM in the diets. Fish, like other animals, eat to satisfy their energy requirements (Wilson 2002; Bureau et al. 2002), and rely majorly on lipids and proteins found in the diet as energy sources, especially in the case of carnivores as carbohydrates do not constitute a major part of their diets in nature (Halver & Hardy 2002). While proteins are mostly used by fish for growth and maintenance (Wilson 2002), lipids are the major energy provider and are often used to spare protein for growth in aquafeeds (Sargent et al. 2002). In the present study, the formulated diets were made to be isoenergetic (DE of 14 MJ/Kg considered enough to provide good growth in *S. rivulatus* based on previous study by Abou-Daoud et al. (2014)), and wheat flour was used as a source of carbohydrates to balance the energy. However, wheat flour inclusion decreased when more SBM was added to the diets, and hence most carbohydrates in treatments D3, D4, and D5 were of SBM origin. Marbled spinefoot is an herbivore and it would be logical to assume that it would utilize carbohydrates for energy. It seems that *S. rivulatus* lacked the ability to

properly utilize SBM carbohydrates, and had resorted to body lipid for energy compensation as lipid content in the body decreased with addition of SBM in the diet while moisture content increased. Similar results were reported by Elangovan & Shim (2000) in juvenile tin foil barb *B. altus*, where carcass fat content in the fish decreased with increased SBM in the diet, one of the possible causes being the increase in proportion of indigestible carbohydrates with increase in SBM. SBM caused a reduction in nutrient deposition and less availability of digestible energy compared to fish meal in sharp snout seabream *Diplodus puntazzo* (Hernández et al. 2007), and was shown to reduce intraperitoneal fat in juvenile sutchi catfish *P. hypophthalmus* (Phumee et al. 2011). Another reason for the decrease in body lipid of fish in the present study could be a reduced dietary lipid digestibility as effect of certain alcohol-soluble components found in SBM (Olli & Krogdahl 1995; Elangovan & Shim 2000). Either or both explanations are plausible in this situation as ANFs present in soy would reduce protein availability, cause possible liver damage and impair metabolism, and decrease lipid digestibility in the diet. Presence of indigestible carbohydrates and decrease in overall digestible energy of the diets would prompt increased use of body fat for energy, which is supported by lack of significant differences in VSI and decrease in body lipid proportion in fish offered diets with increasing SBM inclusion.

D. Hematological Parameters

There were no significant differences observed in total RBC or WBC counts among treatments. Differential counts also did not show any significant changes with SBM inclusion. However, there was an increasing trend in neutrophil counts with increased inclusion of SBM, although no significant statistical differences among

treatments were found, and neutrophil counts in general were lower than those reported for aquacultured *S. rivulatus* earlier by Nasser (2012). Monocyte counts also showed an increasing trend, albeit only up to 75% SBM protein inclusion. Both monocytes and neutrophils had markedly narrower ranges of distribution compared to those previously reported (Nasser 2012), the cause of these differences unclear. In general, increase in neutrophil and monocyte counts with increase in SBM inclusion in the diet is indicative of intestinal inflammatory reaction, as was repeatedly shown in studies discussing SBM-induced enteritis in intestines of fish (Baeverfjord & Krogdahl 1996; Bakke-McKellep et al. 2000; Merrifield et al. 2011). It is possible that *S. rivulatus*, taking into consideration that the species is herbivorous, might have experienced inflammation and then was able to adapt and recover after period of time. Study by Urán et al. (2008) presents evidence of temporary SBM-induced enteritis in common carp (*Cyprinus carpio* L.), as the fish were able to successfully recover from enteritis after 3 weeks of feeding. This would explain the lack in significance among differences in neutrophil counts. Hematology research in Siganids in general and in *S. rivulatus* in specific is largely lacking, and hence any assumption of possible inflammatory reaction occurring in the intestinal tract of the fish in the present study (especially so in absence of any information on gut histology) cannot be empirically corroborated.

Hematocrit levels tended to decrease significantly with increase in SBM inclusion, and fish offered full SBM diets had the smallest PCV among all treatments. Similar effect was reported by Lim et al. (2011) for tiger puffer *T. rubripes*, and the decrease in hematocrit was attributed to anti-nutritional factors present in SBM. Because SBM inclusion in the diets can alter liver morphology and metabolism (Robaina et al. 1995; Lin & Luo 2011), it is possible that SBM inclusion at high levels

in the diets may have affected hematopoiesis in our fish as liver is one of the sites of blood formation in fish (Catton 1951). Lack of nutrient availability resulting from SBM in the diets might also have caused a decrease in hematocrit. Phosphorus deficiency can lead to reduction in hematocrit level in catfish (Lall 2002), which in this case would be the result of phytic acid present in SBM. Hemoglobin, although not significantly different among treatments, tended to decrease when SBM exceeded 50% inclusion in the diet. Hemoglobin is associated with oxygen carrying capacity and high hemoglobin values are characteristic of more active fish (Satheeshkumar et al. 2012), while abnormal or decreased hemoglobin concentrations could be an indicator of anemia (Clauss et al. 2008). Fish in the present study could not have been anemic as PCVs were above 20% in all treatments (PCVs less than 20% are usually associated with anemic condition in teleosts (Clauss et al. 2008)) and no significant decrease in RBC counts was observed, and thus the observed decrease in hemoglobin levels could only be of dietary origin. Liver damage as result of ANFs in the diet could lead to impaired hematopoiesis and thus less hemoglobin. Decrease in HGB could also result from nutritional deficiencies, specifically in micro-nutrients such as iron. Although SBM is quite rich in iron, presence of phytate in soybeans can reduce its availability for the organism, negatively affecting hemoglobin formation (Brown et al. 2008).

Total plasma protein levels showed no significant differences among treatments and did not follow any specific trend. Plasma protein level fluctuations can hint at the relationship between the dietary protein offered and body protein metabolism, and high protein concentration in the plasma could mean poorer protein utilization and increased protein turnover and degradation (Ye et al. 2011). Increased plasma protein could also indicate structural liver alterations and impairment of protein

liver metabolism (Coz-Rakovac et al. 2005). Although TPP levels obtained in this study are higher than those reported earlier by Nasser (2012) for cultured *S. rivulatus*, the possibility of liver damage is improbable because no significant differences were observed among TPP results of fish in all treatments.

E. Conclusion

Results of the present work suggest that SBM is unsuitable as an alternative protein source in diets for *S. rivulatus*. Although statistically soybean meal could be used to substitute fish meal in diets for marbled spinefoot at inclusion levels of 467.2 g/Kg, the decreasing trend in growth with SBM inclusion suggests otherwise. Further investigations into whether soy protein concentrate could be used instead of SBM as a protein source in rabbitfish diets should be performed. Moreover, a study of the effects of dietary SBM on gut morphology, lipid deposition in viscera and liver condition as well as enzymatic activity would probably allow us to better understand the effects of SBM inclusion on fish physiology. Finally, an assessment of the effects of dietary SBM on gut microbiota seems necessary.

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