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RESEARCH ARTICLE

Growth Performance and Body Composition of Zebrafish Fed Plant Protein-Based Diets and a Commercial Diet

Aiyelari T. A[1], Abdul A.S[2]

[1] Department of Fisheries and Aquaculture Management, Faculty of Agricultural Sciences, Ekiti State University, Ado Ekiti Nigeria.
[2] School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom.

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Corresponding Author: Aiyelari T. A, Department of Fisheries and Aquaculture Management, Faculty of Agricultural Sciences, Ekiti State University, Ado Ekiti Nigeria.

Abstract

An eight-week experiment was carried out to compare the effects of SBM and RSM based diets with a commercial fish meal diet on the growth performance and body composition of zebrafish. The study used repeated but non-invasive measurements to evaluate the acceptability and impact of these diets on these fish. This study was conducted according to a 3 x 2 factorial arrangement involving three foods at two stocking densities. Fish were fed three times daily for eight weeks. Morphometric measurements, water quality and feed allowances were determined every week. The proximate analysis was determined according to AOAC methods. The condition factor of the fish and the digestibility of the feeds were determined. The water quality parameters remained within the range recommended for tropical aquaculture. The length, weight and the condition factor were significantly higher ($p<0.05$) in the fish fed commercial diet. There were no significant differences ($p<0.05$) in the length and weight of the fish between the two stocking densities within each dietary treatment. The positive relationship ($R^2 = 0.87$) between the height and weight of the fish fed the RSM diet was more significant than the other two foods. The survival rate ranged between 95.2% and 97.8%. The WG, FCR and PI were significantly higher ($p<0.05$) in the fish fed the commercial diet (0.215 ± 0.01, 0.170 ± 0.12 and 1.449 ± 0.11) than the other foods. There were no significant differences observed ($p<0.05$) in the SGR, FI and PER among the fish fed the three diets. The CP, NFE and the ash were not significantly differences ($p<0.05$) (on the dry matter basis) for the fish fed the diets. However, the ester extract (EE) of the fish fed soybean meal diet was significantly lower ($p<0.05$) (5.697 ± 0.503) compared to others. The moisture contents of the fish ranged between 73.5 % in fish fed RSM diet to 77.0 % in fish fed the SBM diet. There were no significant differences ($p<0.05$) in the energy content of the diets and the fish fed the diets. This study revealed the possibility of soybean and rapeseed to partially replace the high cost of commercial fish meal diet in the culture of zebrafish and other related cyprinids and aquarium species.

Keywords: Plant Protein-Based Diets, Zebrafish, Growth Performance, Body Composition.

1. Introduction

Fisheries and aquaculture are the essential sources of food, nutrition, income and livelihood for people around the world. The efforts in research and development in aquaculture has been directed toward the goal of food security with significant improvement in the recent time. One of the efforts adopted in the current study is to sustainably source for plant proteins as an alternative to highly competitive and expensive fish meal and fish oil (Mazurkiewicz, 2009) and determining their nutritional suitability in the diets (Watanabe, 2002). There is, however, optimism which is further reinforced by a new wave of international collaborations. The sustainable use of

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our aquatic resources can only be achieved through vigorous and combined efforts in technology, human resources and understanding of the nature of the aquaculture industry. The growth of world aquaculture has been stimulated by several factors, including population increases, dietary shifts and advances in aquaculture technology. Diet formulation and manufacture involve the selection and combination of feed ingredients to form a mixture of products that delivers the nutrients needed to meet the production goals in fish farming. This practice can also reduce feeding time and feed waste while improving water quality and environmental safety.

Commercial feeds are expensive as most of the ingredients are imported and prices keep increasing continually. So, finding alternatives to the fish meal is needed to reduce cost and reliance on this valuable and finite source of protein. According to Hardy (2008), one of the methods to develop less expensive and effective formulations is lowering fish meal levels in diets. In Europe, an additional argument supporting the search for plant protein sources for feed is the increasing danger that components of animal origin can transmit pathogens (e.g. the manifestation of mad-cow disease which resulted in reduced use of animal meals in the fodder industry, (Mazurkiewicz, 2009). Increased utilisation of plant protein meals has been embraced as a sustainable alternative to fish meal (Gatlin III *et al.*, 2007; Barrows *et al.*, 2008). Today, the most widely applied alternative plant protein to replace fish meal in aquaculture is soybean meal, mainly the extracted and the protein concentrates (Nordrum *et al.*, 2000), though with little success. There has been the report of partial substitution of soybean meal for fish meal in quantities up to 50 -60% or the application of its by-product (Elangovan and Shim, 2000; Kotzamanis *et al.*, 2008; Mazurkiewicz, 2009). The report also confirmed that the use of such massive percentage in feed was their nutritional (total protein, amino acid composition) and palatable properties (Hasan *et al.*, 1997; Azevedo *et al.*, 1998). However, studies in culture fish have shown that soybean meal induces intestinal inflammation (enteritis) (Hedrera *et al.*, 2013), which indicates that there is a need to research in other available plants (legumes) locally available with a reasonably amino acid profile that can replace fish meal. Plant protein like rapeseed meal, lupin seed meal, sunflower seed meal, peanut or cottonseed meal had also found their application, though with some limitations due to their anti-nutritional factors. The rapeseed has been reported to have high nutritional value, and thus great potential as an alternative protein source for fish nutrition (Adem *et al.*, 2014) The highest fishmeal replacement level of up to 100% have been reported in feeding trial with rainbow trout (*Oncorhynchus mykiss*), 30% substitution of fish meal protein for common carp (*Cyprinus carpio*) (Adem *et al.*, 2014) and 25% substitution of fish meal protein for wels catfish (Slawski *et al.*, 2011) without affecting the growth performance of the fish. It is one of the world-leading edible oilseed crops. It has high protein content, which is distinguishable by a well-balanced amino acid composition and a high biological value (Enami, 2011). The heat treatment of the plant protein reduces the anti-nutritional factors and increase the apparent protein digestibility (Peres *et al.*, 2003).

Zebrafish (*Danio rerio*) formerly known as *Brachydanio rerio* is one of the approximately 45 *Danio* species all over the world (Reed and Jennings, 2010). They are tropical freshwater fish which belong to the minnows and carp family - Cyprinidae of the order Cypriniformes. It is native to Himalayan region and particularly indigenous to Ganges region of India, Pakistan, Bangladesh, Nepal and Burma and commonly inhabit the streams, canals, ditches, ponds, slow-moving or and stagnant water bodies including rice fields (Lawrence, 2007; Mayden *et al.*, 2007; Spence *et al.*, 2008; Sanders, 2009; Reed and Jennings, 2010; Markowski, 2011; Ribas and Piferrer, 2014; Parichy, 2015) . In the field study of zebrafish location, it was reportedly found to be common in shallow water bodies with turbidity level up to about 30 cm, and most often in unshaded site with open water among aquatic vegetation and a silty substratum.

Although, zebrafish (*Danio rerio*) has been used extensively in a number of scientific research and disciplines (e.g. developmental biology and genetics; (Detrich III *et al.*, 1998; Grunwald and Eisen, 2002; Xiang *et al.*, 2009); comparative biological studies (Mayden *et al.*, 2007); toxicology (Hill *et al.*, 2005), and evolutionary theory (Parichy, 2006), aquaculture, immune response, nutrition and growth (Lawrence, 2007; Ulloa *et al.*, 2011; Oyarbide *et al.*, 2012; Hedrera *et al.*, 2013; Ulloa *et al.*, 2013; Ribas and Piferrer, 2014). Their ability as a model for nutrition and aquaculture research has been limited, partly due to lack of standardised, defined diets and husbandry conditions. Many ingredients that are common components of aquatic feed diets contain compounds that alter the physiology and behaviour of an organism (Siccardi III *et al.*, 2009). In the existing feeding procedures for *Danio rerio*, few studies have evaluated the effects of diets on growth and survival of *Danio rerio* (e.g. (Meinelt *et al.*, 1999) indicated that availability or none availability of particular nutrient in the diets could affect normal physiological processes. Also, Gonzales Jr and Law (2013) showed that the use of commercial feed and various feeding regime affects the growth and gonadal development in zebrafish. Thus, the development and growth in fish are influenced by the dietary nutrients.

The standard feeding practice for zebrafish cited in numerous books and publications have centred on the administration of a diet which is primarily from natural source –*Artemia nauphli* and a processed feed, usually a tropical fish flakes as growth progresses (Mullins *et al.*, 1994; Matthews *et al.*, 2002; Westerfield, 2007). The common usage of flakes in zebrafish diets is even problematic because it is difficult to determine the precise ingredients and the nutritional profile of such feeds. These kinds of food had been reported to contain potentially harmful; compounds such as genistein and a soy isoflavone which is shown to be an estrogen mimic in mammals and fish (Ng *et al.*, 2006; Sassi-Messai *et al.*, 2009). The practices of feeding and feed management have numerous and critical implications for the success of the normal zebrafish and aquaculture operation. Despite this fact, the optimal feeding frequencies for post-larval zebrafish have never been formally determined. Zebrafish is chosen for this study because of its many advantages over other teleosts. It is easy for handling and breeding experimentation, has short generational intervals and produce large numbers of eggs per brood (100-200 eggs/clutch) (Ulloa *et al.*, 2014), this allows performing all analysis with a considerable number of specimens per data point and its steady availability for genetic manipulation (Kimmel *et al.*, 1995).

The central hypothesis of this research is to test the efficiencies of plant-based formulated diets as a positive replacement of commercial diets on the growth performance and body composition of zebrafish as a model organism. The study used repeated and non-invasive methods for morphometric measurement using a digital calliper and cameral imaging to determine the condition factor. The project aims to evaluate plant protein-based diets for their acceptability and their effects on growth parameters and to compare the effect of SBM and RSM based diets with a commercial fish meal on the body composition of zebrafish. It also evaluated the interactive effects of stocking density and the diets on the body measurement and growth of *Danio rerio*.

2. Material and Method

2.1 The experimental System and Fish

The juvenile zebrafish (AB strain) were acquired from the zebrafish unit of comparative biological centre (CBC), Newcastle University. A total of 50 adult wild type zebrafish were used in a 1:1 ratio of a male to a female in each 1-L tank, and a total tank density of 2 fish per spawning tank was maintained to breed the fish. Each breeding tank consisted of a false-bottom tank containing a mesh screen bottom that was seated within a relatively larger tank to allow separation of released eggs and milt. The embryos were collected and rinsed with a 60-ppm saline solution containing 1mil/L methylene blue. The methylene blue was used for cracking the eggs to facilitate hatching, identified the unfertilized eggs and to prevent fungal and bacterial infestation of the eggs). Fertilised embryos were placed in a culture dishes at a density of 30-50 embryos per dish and incubated at 28 ^{o}C. After five dpf, the larvae were placed in the 2-L tank and were fed on commercial diets, and size ranged from ZM-000 - 200 fry food for approximately six weeks and after that fed tropical fish flakes (ZM: Fish Food/www.ZM system.co.uk). At sixty-two dpf, fish were distributed into the experimental tanks, in an experimental system consists of eighteen rectangular tanks 3-L water capacity tanks (31.5cm x 13.5cm x 13.0cm in dimension) with a common water source, connected to an aquarium pump (Surpass air pump – Resun® Air-8000, China), which supply oxygen continuously throughout the experiment to reduce the accumulation of nitrogenous waste in the tank water. Twelve fish were stocked per tank, and eighteen tanks were used for the 3 x 2 factorial experimental arrangements. A light: dark photoperiod of 14;10 hours was provided with indirect fluorescent lighting. The water quality parameters, which includes dissolved oxygen, pH, ammonia, water hardness, chlorine, nitrites (NO_2) and nitrates (NO_3), were determined weekly. Dissolved oxygen was measured by using dissolved oxygen kit (CHEMetrics Inc. VA 22728, USA). The temperature was monitored daily using an aquarium thermometer (Pond planet, Cleveland, UK). The pH, ammonia, nitrate and nitrite, chlorine, and water hardness were monitored weekly using an aquarium Tetra 6 in 1 water test strip (Tetra GmbH, Germany). All fish were maintained at 26^{o}C, pH 6.8 and 370 µS/cm conductivity in a recirculating system.

Table 1. *Composition of the dietary ingredients.*

Ingredients	CFM diet	SBM diet (g/kg)	RSM diet (g/kg)
Fish Meal (64%[a])	-	229	240
Rape Seed Meal (37%[a])	-	-	240
Soybean Meal (50%[a])	-	229	-
Wheat gluten (60%[a])	-	229	240
Yellow Maize	-	66	53
Wheat Bran	-	65	53
Ground Brown Rice	-	65	53
Lysine (Lys)	-	10	10
Methionine	-	12	12
$CaCO_3$ (Ca^{2+})	-	3	3
Salt	-	4	4
Vitamin Premix	-	15	15
Mineral Premix	-	10	10
Cod-liver oil	-	58	65
(Cr_2O_3)	-	5	5
Proximate composition of the diets			
Dry matter	92.89 ± 0.54	91.65 ± 0.89	92.58 ± 0.58
Crude protein	44.81± 0.24	47.48 ± 1.70	44.30 ± 0.30
Crude fat	12.59 ± 0.88	10.98 ± 0.35	11.78 ± 0.33
Crude fibre	3.15 ± 0.61	7.58 ±0.66	10.97 ± 0.43
Ash	3.39 ± 0.04	6.85 ± 0.79	7.11 ± 0.14
Nitrogen-free extract	28.41 ± 0.47	18.41 ± 1.21	13.90 ± 0.74
Mineral composition (% DM)			
Calcium	5.00 ± 0.05	8.08 ± 0.06	8.53 ± 0.10
Potassium	1.40 ± 0.07	1.89 ± 0.06	1.05 ± 0.04
Magnesium	0.52 ± 0.00	0.60 ± 0.01	0.55 ± 0.01
Sodium	1.61 ± 0.02	1.28 ± 0.02	1.41 ± 0.01
Phosphorus	0.20 ± 0.00	0.20 ± 0.00	0.19 ±0.00
Zinc	0.03 ± 0.00	0.22 ± 0.00	0.26 ± 0.00
Iron	0.11 ± 0.00	0.40 ± 0.00	0.37 ± 0.00
Amino acids[1] composition g/kg of protein			
Histidine	7.40 ± 1.30	3.78 ± 0.09	3.85 ± 0.20
Leucine	50.73 ± 0.90	66.55 ± 2.76	68.12 ± 3.69
Isoleucine	24.06 ± 1.30	21.62 ± 0.47	17.91 ± 1.95
Lysine	62.44 ± 14.68	33.18 ± 1.03	30.35 ± 1.42
Methionine	5.48 ± 0.53	11.63 ± 1.14	12.46 ± 0.64
Phenylalanine	50.49 ± 2.63	40.31 ± 6.24	39.32 ± 3.50
Threonine	2.54 ± 0.41	2.60 ± 0.24	2.54 ± 0.34
Valine	13.54 ± 1.13	11.94 ± 0.38	10.03 ± 1.30

The essential amino acid only.

2.2 The Diets and Preparation

Dietary treatment evaluated consisted of one most used, commercially available and two laboratory prepared diets. The commercial diet used was TetraMin Tropical Flakes® (47% protein, 10% fat, 3% fibre) (Tetra Holding GmbH, Germany). The laboratory prepared diets were formulated with two different protein sources (soybean meal containing 47% protein, 10.98% fat, and 7.58% fibre and rapeseed meal containing 44.30% protein, 11.78% fat and 10.97 fibre respectively). The ingredient composition of the laboratory prepared diets used are presented in Table 1.

Feed ingredients were acquired from A-One Feed Supplements Ltd, Thirsk, North Hill, Dishforth

Airfield YO7 3DH Thirsk - Dishforth, North Yorkshire, England. Ground brown rice, cod liver oil, NaCl (salt) were purchased from Holland and Barrett, Nuneaton, Warwickshire, UK), Chromium III oxide ($Cr_2O_{3)}$ (Sigma Aldrich). The dried ingredients were ground to fine particles with a coffee grinder (DēLonghi KG49, UK) and thoroughly mixed by Kenwood KM 336 (Kenwood, UK). The ground ingredients were sieved by using Laboratory test sieve with 250 µm aperture (281716, ENDECOTTS LTD, London, UK) to obtain uniform particle size for the juvenile fish diet. Samples of the diets were analysed for their proximate composition, minerals and fatty acid profile. The ingredient mixture was made into a slurry by mixing with about 250 ml warm water. The semi-liquid mixture was thinly spread inside an aluminium foil tray and kept in the freezer at -20 ^{O}C until freeze-dried for five days to a constant weight. Feeding of the experimental fish was accomplished manually. The fish were fed a quantity of feed worth 5 % of their body weight. Feeding of the fish was carried out three times daily (6 – 8 am, 12 – 14 pm and 18 – 20 pm) following the published literature ((Kasiri *et al.*, 2011; Nekoubin *et al.*, 2013). The feed administered was adjusted based on the weight of the fish. However, overfeeding was avoided to prevent fish from getting too fat, disease infection and to maintain water quality.

2.3 Morphometric Measurements of Fish

Each fish group was assessed weekly for its length and weight. The weight measurement was used to adjust the fish feeding allowance (as described above) and for monitoring the growth of the fish. This assessment was accomplished by weighing all the fish in a tank as a unit to the nearest 0.01mg. One fish was randomly selected from each tank and euthanised with tricaine methanesulphonate MS-222 (Sigma) in 100 ml aqueous solution at 200 mg/L as used by Matthews *et al.* (2002) for morphometric measurement. Each euthanised fish was then placed in a petri dish with good background and photographed with a digital camera (Samsung ES90). Image J and MATLAB computer software were used to obtain the measurement of both length and width from the captured images with the camera. A digital calliper (Silverline®, 380244, London UK) was also used to measure the length and width of the fish. All length measurement recorded to the nearest 0.01mm. The tail and mouth ends were acquired as optimal points to describe the total length of the fish as the fish share the same genetic characteristics concerning their morphology. At the end of the experiment (118 dpf), each fish was euthanised and then measured for its weight and length. The measurements of both length and weight were used to determine the condition factor (the well-being of the fish) according to Fulton condition factor (Fulton, 1902).

2.4 Growth Performance

Various measurements and growth performance indices were evaluated. The weight gain was calculated as the final weight (g) – the initial weight of the fish per tank (g). MWG/Day = mean weight gain (g) / number of days. The specific growth rate (SGR) = (ln w_2 - lnw_1) / (t_2 - t_1) x 100. Where: W_1 = weight of fish at the beginning of the experiment (t_1 days), W_2 = weight of fish at the end of the experiment (t_2 days), ln = natural logarithm and $t_2 - t_1$ = experimenter periods in days. Total feed intake was calculated as the cumulative food consumed by each fish group per tank. The FCR was calculated according to Houlihan *et al.* (2001) from the relationship between food intake and weight gain by fish. (i.e.) FCR = food intake (g) / weight gain (g). The protein efficiency ratio (PER) = net weight gain (g) / protein intake in term of the nitrogen content of the feed (g). Daily protein intake (g) was computed by the relationship between the protein intake and the number of days of the experiment. DPI = protein intake/number of days. The energy was determined by using an adiabatic bomb calorimetric (Gallenkamp auto bomb, CBA-305-010M, UK) with a benzoic acid standard. The result was cross-referenced with energy determination using the conversion factor (FAO, 2003). The mathematical formula determined condition factor according to Fulton condition factor (Fulton, 1902), $K_{FL} = W/L^3 \times 100$, where W= fish weight (mg), and L= fish total length (mm) while K is the coefficient of condition.

2.5 Body Composition

The proximate composition of the fish was performed at study completion by the application of AOAC (2005) official method. However, the Crude protein was determined by using the cube method (Elementar Vario Macro cube, Germany), while the fat content was extracted by using dichloromethane (98.9%) (Sigma Aldrich, UK), a modified method of Folch *et al.* (1957). All the analysis was conducted in triplicates. Before the analysis, euthanised fish samples were freeze-dried (VirTis SP Scientific. NY, USA) after pre-freezing which allowed the samples to exhibit a crystalline structure at -20 ^{o}C when the fish was frozen below the eutectic temperature. The fish samples were grounded with a coffee grinder (DēLonghi Living innovation, UK). The fatty acids were characterised by methylation and esterification of the fatty acids following solvent extraction and analysed by using GC-MS machine. The GC-MS analysis of the derivatised organic acid compounds was performed on an Agilent 7890A GC split/splitless injector (280°C) linked to an Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800V, interface temperature 310°C). The analysis of minerals was accomplished by using inducible coupled plasma – optical emission spectrometry (Vista – MPX CCD Simultaneous ICP – OES, USA) after acid hydrolysis of the samples. Also, the analysis of the amino acids was accomplished by derivatisation using EZ-Faast technology and kits (Phenomenex, Torrance, CA) after Hydrochloric acid hydrolysis under anaerobic condition by boiling for 24 hours in an oil bath at 110°C.

2.6 Statistical Analysis

Data collected were subjected to Normality test for normal distribution using IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. When the data obtained from measurement were found to be significantly different from a normal distribution, a non-parametric analysis equivalent was employed. Data that were not significantly different from normal distribution were subjected to analysis of variance (ANOVA) and student two samples T-test. The results were reported as means ± SD. The correlation coefficient (r) of the length and weight relationship of the fish was determined by using a scattered plot. The effects of the diets, stocking density and the interaction of the diet with the stocking density were determined by GLM (general linear model). The effects of stocking density and diet in growth on a weekly basis was studied. The Mauchly's test of sphericity and the Levene's test of equality of variation were used to analyse the differences in the growth of the fish weekly. Tukey's post-hoc test was used to compare mean to observe significance at $p < 0.05$.

3. Results

The results of the percentage survival of the fish at the end of the experiment are presented in Fig.1. The percentage of survival ranged between 95.8 % in the fish fed SBM diet to 97.2 % in the fish fed CFM and RSM diets, respectively. The high survival rate of zebrafish fed the plant protein-based diet compared to those fed commercial FM-based diet revealed the possibility of feeding zebrafish with a high percentage of plant protein-based diet without adverse effects on their survival.

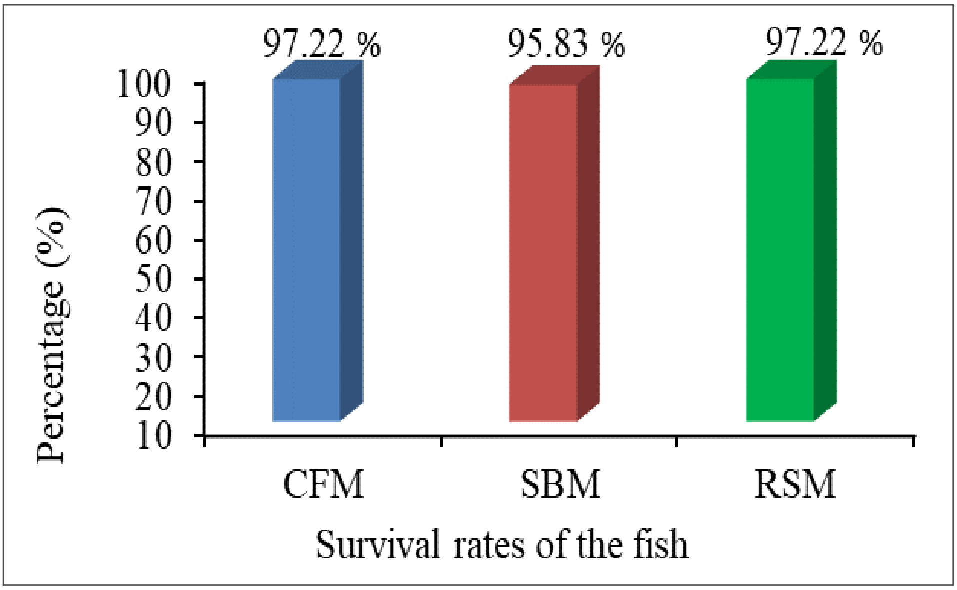

Figure 1. *The survival rates of the fish fed the commercial fishmeal (CFM) and plant protein-based; soybean meal (SBM) and rapeseed meal (RSM) diets, respectively.*

3.1 Growth Performances of Zebrafish Fed the Three Diets

The results of the weekly growth pattern, based on the three diets and the two stocking densities are presented in FIG.2. The fish fed the CFM diet recorded the highest growth. All the fish exhibited a similar pattern of growth irrespective of the diet fed. At the initial stage, the weight dropped in the fish fed SBM and RSM diets (Fig. 1 B & C). After acclimation to the new diets and the environment, the growth patterns of the fish changed. A similar trend was observed in tanks with low and high fish stocking density among the fish fed the three diets.

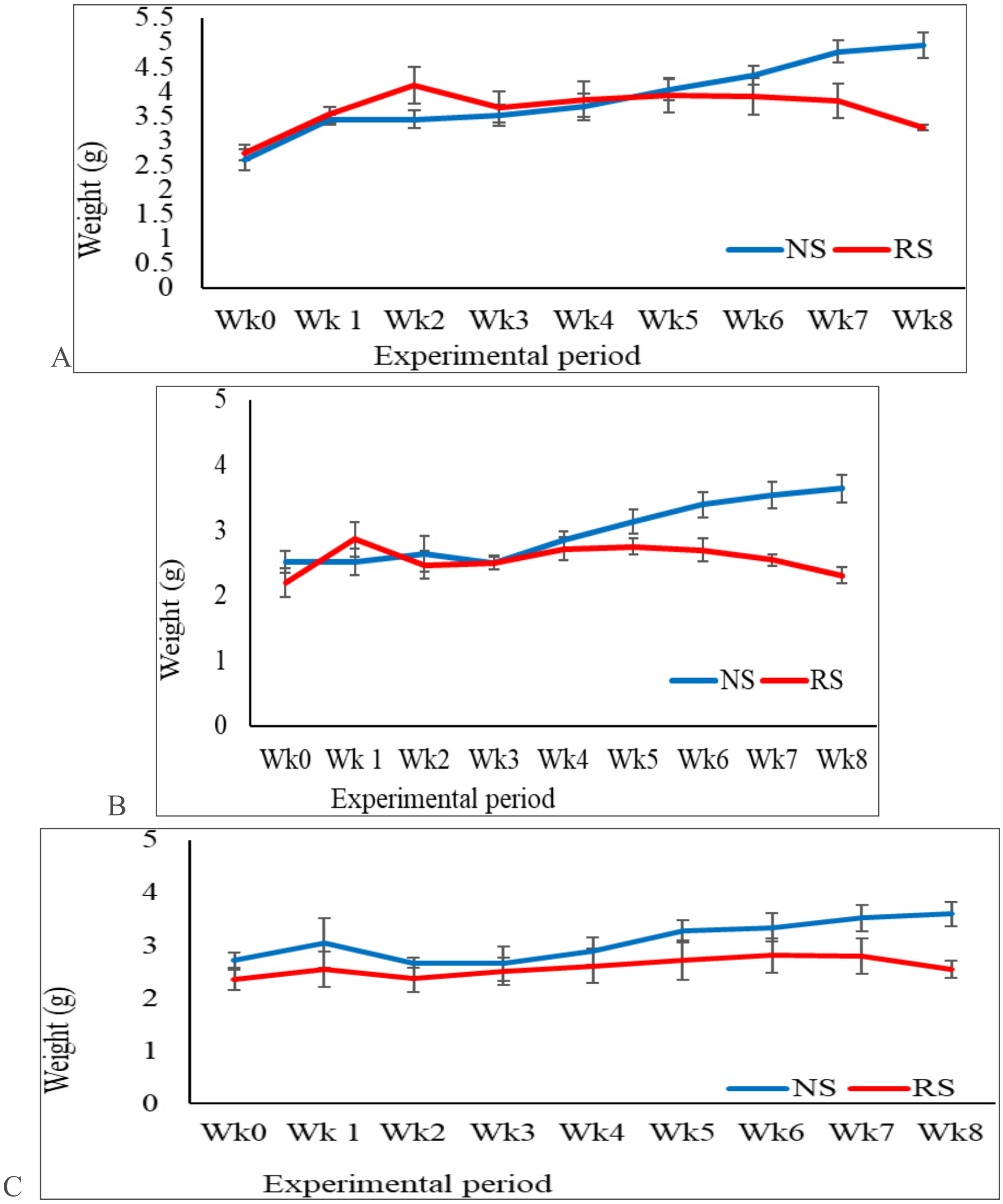

Figure 2. *The weight measurement of the fish fed various diets weekly at two stocking densities. A is the fish fed commercial fish meal-based diet, B is the fish fed soybean meal-based diet, and C is the fish fed rapeseed meal-based diet. NS = high stocking density, RS = low stock density.*

The multivariate test results show that there were significant differences ($p<0.05$) in the weight of the fish based on the stocking density. However, there was no significant difference observed among the fish fed the diets. The Mauchly's test revealed no significant differences in the weight of the fish relative to the interaction between the diets and the stocking density.

The Mauchly's test of sphericity equally revealed significant differences ($p<0.05$) in the weight of the fish between the first week and the second week of the feeding trial. There were no significant differences; however, in the weight of the fish between the second, and the third week until the fourth week through to the seventh week where significant increments ($p<0.05$) were observed. Significant differences manifest in the weight of the fish between the two stocking densities from the fifth weeks to the eight weeks. The difference was due to the reduction in the numbers of the fish relative to the respective culture medium. Levene's test of equality of error of variances across the groups from one week to the other showed that there were no significant differences ($p>0.05$) in the error variances of the weight of the fish. On the overall performance of the fish, the results show a significant interaction of the diet and the stocking density on the weight gain, specific growth rates, food conversion ratio, and the protein efficiency ratios of the fish during the period Fig 3.

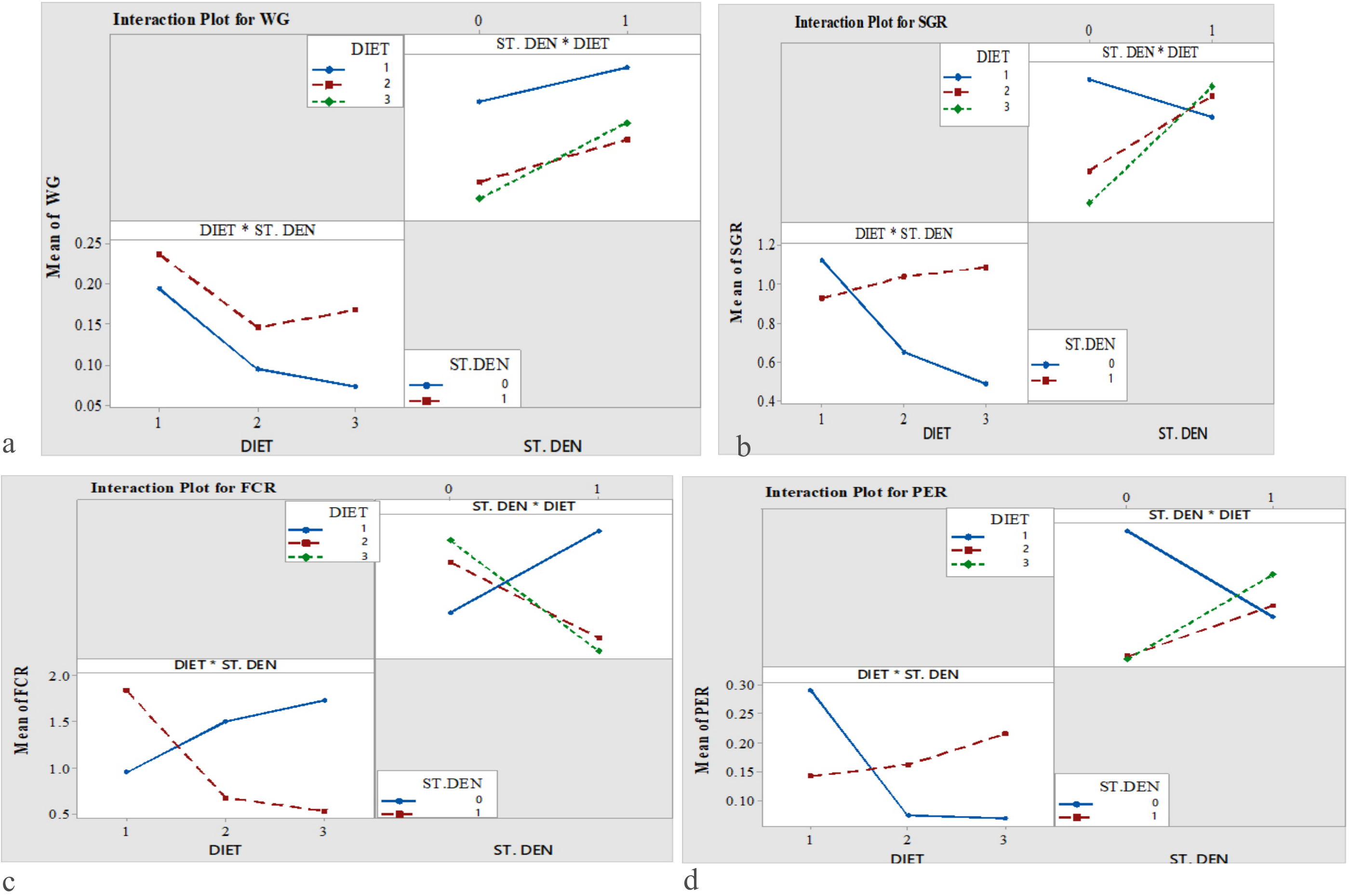

Figure 3. *The interaction of the diet and stocking density on some specific growth parameters of zebrafish fed commercial fish meal and plant protein-based diets.*

3.2 Zebrafish Nutrient Utilisation and Growth Performance

The results of the growth performance of the fish fed the three diets, and the respective stocking densities are presented in Table 2 and 3, respectively. The results of the ANOVA show that there were significant differences ($p<0.05$) in the weight gain (WG), protein intake (PI) and better food conversion ratio (FCR) among the fish fed CFM, SBM and RSM diets. The values were significantly higher in the fish fed the CFM than the other two diets. However, there were no significant differences observed ($p<0.05$) in the SGR, FI and the PER in the fish fed the three diets.

Table 2. *Mean growth performance indices of the experimental fish*

Variables	CFM (n=6)	SBM (n=6)	RSM (n=6)	Total fish	
				NS (n=9)	RS (n=9)
Ini. Wt (g)	2.11 ± 0.25	1.90 ± 0.29	2.05 ± 0.31	2.61 ± 0.09^{a}	1.42 ± 0.07^{b}
Fin. Wt (g)	4.10 ± 0.40	2.98 ± 0.32	3.07 ± 0.27	4.06 ± 0.25^{a}	2.71 ± 0.16^{b}
WG (g)	1.99 ± 0.16^{a}	1.08 ± 0.06^{b}	1.03 ± 0.11^{b}	1.45 ± 0.23	1.28 ± 0.10
FI (g/d)	0.17 ± 0.01^{a}	0.12 ± 0.01^{b}	0.11 ± 0.01^{b}	0.15 ± 0.01	0.12 ± 0.01
FCR	0.09 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.09 ± 0.01
SGR (%/d)	1.18 ± 0.06^{a}	0.85 ± 0.10^{ab}	0.79 ± 0.14^{b}	0.76 ± 0.10^{b}	1.13 ± 0.05^{a}
PI (g)	1.45 ± 0.11^{a}	1.12 ± 0.08^{ab}	0.92 ± 0.09^{b}	1.29 ± 0.09	1.03 ± 0.10
PER	1.38 ± 0.07	0.99 ± 0.08	1.17 ± 0.17	1.07 ± 0.11	1.29 ± 0.09

Values are means ± standard error. The means with the same superscript along the same row are not significantly different at ($p>0.05$). NS = High stocking density, RS = Low stocking density, FCR = Food conversion ratio, SGR = specific growth rate, NA = Not applicable 'n' = the number of replicates. CFM, SBM, and RSM are the fish fed the various diets. There are 56, 55, and 56 fish samples for each of the six replicates under each dietary treatment, respectively.

The fish fed CFM diet converted more food to flesh and gained more weight than those fed other diets. Likewise, fish under the low stocking density gained more weight significantly ($p<0.05$) than the fish in tanks with high stocking density. The protein intake was significantly higher in the fish tanks with high stocking density than those with low stocking. There were no significant differences ($p>0.05$) in the SGR, FCR, FI, as well as the PER between the two stocking densities among the fish fed the three diets. Fish in tanks with low stocking density converted more food to flesh and gained more weight than those stocked at high density. The two-sample T-test results show that there were no significant differences between the two stocking densities in the fish fed CFM diet with respects to all the growth indices except the weight parameters. However, the results were different in the fish fed either SBM or RSM diets. The WG was similar in both stocking densities for the fish fed both SBM and RSM based diets. The PER and SGR were higher fish with low stocking density among those fed RSM and SBM diets. However, FI, FCR, and PI were not significantly different ($p>0.05$) in fish relative to both high and low stock densities. The SGR was higher in the low stocking density than those of high stocking density.

Table 3. *Comparison of the growth performance indices among the fish fed the various diets based on stocking density, and (Diet x stocking density interaction)*

Variables	CFM		SBM		RSM	
	NS (*n* = *3*)	RS (*n* = *3*)	NS (*n* = *3*)	RS (*n* = *3*)	NS (*n* = *3*)	RS (*n* = *3*)
I. Wt (g)	2.61 ± 0.21[a]	1.61 ± 0.09[b]	2.51 ± 0.17[a]	1.28 ± 0.13[b]	2.72 ± 0.14[a]	1.37 ± 0.11[b]
F.Wt (g)	4.94 ± 0.26[a]	3.26 ± 0.06[b]	3.64 ± 0.21[a]	2.31 ± 0.12[b]	3.60 ± 0.24[a]	2.55 ± 0.16[b]
Wt.G (g)	2.33 ± 0.05[a]	1.65 ± 0.10[b]	1.13 ± 0.14	1.03 ± 0.03	0.88 ± 0.01	1.17 ± 0.01
SGR (%)	1.13 ± 0.05	1.25 ± 0.10	0.66 ± 0.08[b]	1.04 ± 0.09[a]	0.49 ± 0.08[b]	1.09 ± 0.05[a]
F.I (g)	0.19 ± 0.01	0.16 ± 0.02	0.15 ± 0.00	0.11 ± 0.01	0.12 ± 0.01	0.09 ± 0.01
FCR	0.90 ± 0.06	0.66 ± 0.07	1.36 ± 0.32	0.74 ± 0.09	0.15 ± 0.04	0.08 ± 0.01
PI (g)	1.59 ± 0.09	1.31 ± 0.17	1.26 ± 0.04	0.99 ± 0.13	1.04 ± 0.10	0.80 ± 0.12
PER	1.48 ± 0.06	1.29 ± 0.12	0.90 ± 0.09	1.07 ± 0.13	0.84 ± 0.12[b]	1.51 ± 0.15[a]

Values are mean ± standard error. The mean values with the same superscript in the same row under the same diet are not significantly different (p>0.05). NS = high stock density, RS = low stock density, n = the number of replicates. SGR = specific growth rates, PI = protein intake, and PER = protein efficiency ratio. CFM, SBM and RSM are the fish fed the various diets.

3.4 The Chemical Composition of the Fish Fed the Three Diets

The results of the proximate composition of the fish fed the CFM, SBM and RSM based diets are presented in Figure 2, The results show that there were no significant differences ($p>0.05$) in the dry matter (25.92 ± 1.80, 23.05 ± 1.66 and 26.53 ± 1.36), moisture contents (74.08 ± 1.8, 76.95 ± 1.66 and 73.47 ± 1.36), crude protein (13.78 ± 0.99, 13.19 ± 0.98 and 14.50 ± 0.76), ash contents (1.09 ± 0.32, 1.20 ± 0.28 and 1.61 ± 0.22) and the nitrogen-free extract contents (3.05 ± 0.31, 3.04 ± 0.28 and 2.95 ± 0.42) for the fish fed CFM, SBM and RSM respectively. However, the ether extract of the fish fed SBM was significantly lower (5.70 ± 0.50) than that of the CFM and RSM (8.46 ± 0.61 and 7.35 ± 0.60 respectively).

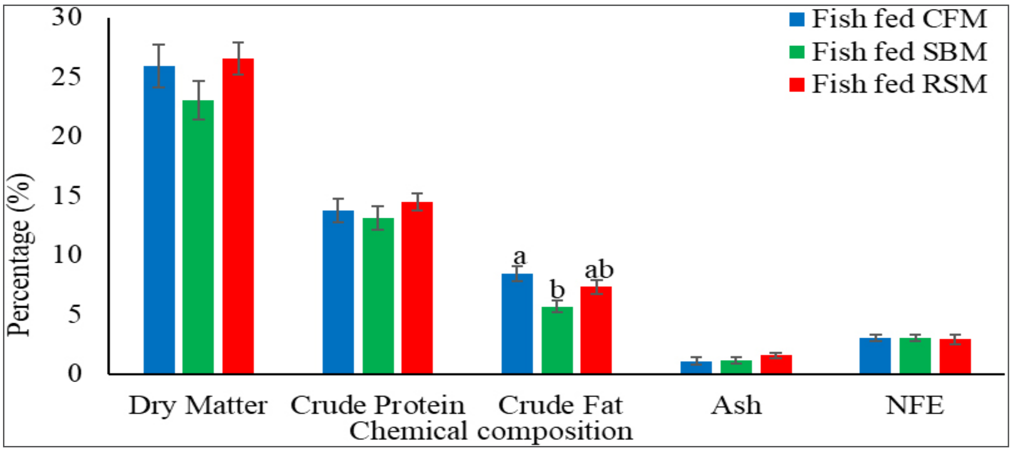

Figure 4. *The chemical compositions (% DM) of the fish fed CFM, SBM, and RSM based diets. Data for crude protein, ether extract, ash, and NFE are based on the DM. NFE = Nitrogen free extract. Bars with the same letter are not significantly different (p>0.05). The fat content was lowest in the fish fed SBM.*

4. Discussion

The fundamental approach to establishing a standard diet for any particular fish species considered a model organism begins with several tests of diet acceptability, mainly when the formulation is comparison-based with a diet compounded with highly competitively used, and expensive fish meal and fish by-products. Fish, accept a feed based on its availability, chemical and physical characteristics, the nutritional value, the environment, stressors and social interactions, and method of food delivery (Houlihan *et al.*, 2001; Lawrence, 2007; Eriegha and Ekokotu, 2017). Various research has shown the possibility and recommendation of cost-effective plant protein sources for the formulation of feed for tropical fish culture with varying success of survival (Tacon and Metian, 2009; Kaushik and Troell, 2010; Geay *et al.*, 2011; Radhakrishnan *et al.*, 2016; Al-Thobaiti *et al.*, 2018). The results of the survival rates in this study ranged between 95.83 % and 97.3 % in the feeding experiments (Fig. 1), confirming zebrafish as a euryphagous species with the ability to consume wide varieties of available food substances and convert those into body flesh (Watts *et al.*, 2013; Ulloa *et al.*, 2014). The results are similar to the finding of Silva-Carrillo *et al.* (2012), who reported a survival rate above 95% for juvenile spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869) fed SBM. Al-Thobaiti *et al.* (2018) reported that 50 -100 % survival for Tilapia (*Oreochromis niloticus*) fed plant protein-based diets. A survival rate of 87 % was reported for zebrafish fed compound feed from the first feeding onward (Kaushik *et al.*, 2011). The survival rates of zebrafish were higher than the mean survival ranging from 55 – 86 % reported at 27 dpf (Carvalho *et al.*, 2006), and 76 % for zebrafish fed TetraMin Tropical flakes (Siccardi III *et al.*, 2009). The results show that there were no adverse effects on the survival of zebrafish when fed both SBM and RSM based diets. The results agree with the findings of Siccardi III *et al.* (2009) and Smith Jr *et al.* (2013) who observed no significant differences in the overall health condition and survival of *Danio rerio* fed different laboratory dietary treatments as determined by the analysis of their condition factor.

Many environmental factors such as temperature, stock density, and water quality as well as the genetic factors affect the growth and maturity of fish (Zheng *et al.*, 2012; Singleman and Holtzman, 2014; Holtzman *et al.*, 2016; Mansano *et al.*, 2017). Besides, fish growth is influenced by body weight and feed consumption alongside the precocity of distinct sexes of fish observable in the growth of different tissues and physiological state of the animals (Dumas *et al.*, 2010; Mansano *et al.*, 2017). Growth is a quantitative trait which may be measured at different stages of an animal's life cycle (Ulloa *et al.*, 2011), with many variations occurring in the growth rates during laboratory experimentation. Several studies have been conducted to assess growth of zebrafish relative to nutrition and maturity (Singleman and Holtzman, 2014), influence of protein source on body composition (Smith Jr *et al.*, 2013), growth and survival (Siccardi III *et al.*, 2009), influence of diets on eggs and larvae quality (Utomo *et al.*, 2008), nutrition and health (Watts *et al.*, 2016), and nutritional requirement (Zhu *et al.*, 2009; Fernandes *et al.*, 2016).

The increased weight of zebrafish fed the various experimental diets was an indication that zebrafish accepted the diets and were able to convert the dietary protein into flesh. There was a lack of significant differences ($p>0.05$) in the SGR, FI, and PER. However, the fish fed CFM diet recorded higher WG, PI and better FCR than the others fed laboratory defined diets formulated with either SBM or RSM. The differences can be attributed to the variations in the specific source of nutrients between the diets which could have affected the physiological or molecular mechanisms that influence the weight of the fish fed the undefined CFM based diet (Siccardi *et al*., 2009) and longer habituation of the fish to the commercial diet than the other groups fed either SBM or RSM based diets. The high weight gain obtained in the fish fed CFM can also be attributed to the acclimation period for the fish to adapt to new diets and some anti-nutritional factors inherent in RSM and SBM (Akande and Fabiyi, 2010; Kaur *et al.*, 2012; Sogbesan *et al.*, 2017), that could inhibit the nutrient utilisation and affect animal's growth (Burel *et al.*, 2000; Matthaus, 2004; Enami, 2011). Estimated values of 5 – 16 g/kg DM has been reported as tannin contents of ingredient used in animal feeds. It was reported to be highest in oilseeds, followed by legumes (Collins *et al.*, 2013; Omnes *et al.*, 2017). This factor might have played an essential role in the reduced growth rate observed in the fish fed both SBM and RSM diets. The high feed intake might have also contributed to the weight differences between the treatments as fish were fed based on their body weight. The growth and FCR of fish are leading indices for the determination of the acceptability and consumption of artificial diets. The FCR is mostly influenced by the VSI, homogeneity, genetic factor, feeding, and well-being of a fish (Robinson and Li, 2015; Eriegha and Ekokotu, 2017; Hasan and Soto, 2017). It measures averagely 1.4-1.8 for omnivorous species. The FCR generated in this study from experiment 2, and 3 ranged from 0.05 - 0.11. The FCR recorded in this study were low compared to the reports of 2.8 to 1.2 for Salmon (Hasan and Soto, 2017), lower values ranging from 0.9 to 1.3 were reported for Trout, Salmon, and Tilapia (Craig *et al.*, 2017). This low value compared to the recommended can be attributed to the small size of the fish, high protein digestibility, and improved culture management (Hasan and Soto, 2017). The specific growth rates are one of the most frequently used functions in fisheries and aquaculture for biological interpretation of the growth per day. The SGR in this study was similar to the results obtained by Chambel *et al.* (2015) that stocking density and diet have effects on the SGR, condition factor and fish survival. The lack of significant variation in the nutrient utilisation between the two stocking densities in chapter five can be attributed to the schooling behaviour of zebrafish (Montero *et al.*, 1999; Costas *et al.*, 2008). This study showed that SBM and RSM fed fish at lower density gained more weight, had higher SGR, better FCR, and PER than those at high density. The study agrees with the findings of Vargesson (2007) on zebrafish, Oppedal *et al.* (2011) on *Salmon salar,* Alhassan *et al.* (2012) and Costa *et al.* (2017) on *Oreochromis niloticus*, that stocking densities influenced the growth and performances of fish. The study revealed that diet and stocking density played a vital role in the specific growth performance of zebrafish.

The lower FCR in the fish fed CFM is an indication of better food conversion than those fed both SBM and RSM based diets. The FCR of fish and other aquatic smaller animals has been reported to be affected by many factors among which are the water quality, rate and manner of feed presentation to the fish, and health status of the fish (USAID, 2011). None of these factors was significant enough to affect the FCR in this present

Although recent attention has been given to zebrafish for its potential as a model for genetics and different diseases in human beings, few studies have reported its body composition outcome relative to different diets. The various studies that demonstrated and quantified the body composition of fish adopted different approaches based on the environment, fish species and technologies. Smith Jr *et al.* (2013) applied quantitative magnetic resonance (QMR) to establish body composition based on the lean and fat mass of zebrafish. González *et al.* (2006) studied yellow perch; Bolasina and Fenucci (2007) used Brazilian codling (*Urophycis brasiliensis* Kaup, 1858); Chukwu and Shaba (2009); Siddique *et al.* (2012) studied marine fishes; and Suseno *et al.* (2014) used pelagic fish (*Amblygaster sirm* and *Sardinella gibbosa*); all these studies applied AOAC methods. Also, Breck (2014) reviewed the body composition of fish from literature and generated a linear functional relationship between fish body lipid and body water. This present study applied the AOAC (2005) methods to investigate the effect of SBM and RSM based diets on the body composition of zebrafish. The results in this study were in the range reported for various tropical fish species (Murray and Burt, 2001; Ali *et al.*, 2006; Mazumder *et al.*, 2008; Kaushik *et al.*, 2011; Alfa *et al.*, 2014; Bogard *et al.*, 2015; Bernardes *et al.*, 2016; Taşbozan *et al.*, 2016). However, the CP in this study was lower than that reported for *Clarias gariepinus* (18.34 - 20.32 %) and Tilapia species (18.65 – 18.74%) (Osibona *et al.*, 2009; Abimbola *et al.*, 2010) respectively. This study showed that diet affected the protein composition of the zebrafish.

The fish muscle constitutes 50-60 % muscle (Coppes Petricorena, 2015) of which the protein constituent is about 16 - 21 % and lipids of about (0.5 – 2.3 %). The ether extract recorded in this study placed zebrafish as high-fat fish (despite being a small fish) compared to the findings of Mazumder *et al.* (2008) for some small fishes of Bangladesh. According to Coppes Petricorena (2015), lipid and water are connected components in fish: hence, as lipid increases, the moisture content decreases as equally observed in this study. The varied composition recorded for different species relative to this study may be attributed to environment and feeding factors (Bogard *et al.*, 2015; Antony, 2016). The results of the ash content of zebrafish in this study are similar to the report of Begum *et al.* (2012) and Bernardes *et al.* (2016). It is also in the range reported by Bogard *et al.* (2015), but lower than the findings of Njinkoue *et al.* (2016) who reported 7.17 and 7.28 % ash contents for Pseudotolithus typus and Pseudotolithus elongates respectively. The diets had no significant effect on the protein and the carbohydrate (NFE) levels of the fish but significantly impacted the fat (FA), and ash (mineral) composition of the fish. The manifestation of the effect of the diets on the fat content of the fish was observed. The results could be attributed to the low-fat content of the SBM based diet in the feed compared to other ingredients.

The fish body is constituted by four significant components often determined in proximate analysis viz: water (moisture), lipids (FA), ash (minerals), and protein (AA). These components constitute about 96– 98 % of the total tissue of fish. Growth of fish is much related to this four-component alongside the carbohydrates, vitamins, nucleotides and other non-protein nitrogenous compounds which were present in variable and small quantities but played vital roles in the body maintenance, growth, and development of the organisms. In general, the results of this study agreed with previous findings that there were no adverse effects of the utilisation of plant protein-based diet on the growth of zebrafish.

5. References

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