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Impact of the partial replacement of fish meal with a chloroplast rich fraction on the growth and selected nutrient profile of zebrafish (*Danio rerio)*

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ABSTRACT

In recent years increasing aquaculture production combined with a high variance in availability and cost of fishmeal and fish oil commodities, has led to a need to identify alternative source materials for protein and the polyunsaturated fatty acids (PUFAs) which fish oil is prized for containing. Chloroplasts are the organelles in plants' leaves where many of the valuable nutrients, fatty acids (FAs), amino acids, vitamins and pigments, are synthesised. Chloroplasts could be incorporated into fish diets either retained in, or liberated from, plant cells. In this study zebrafish were fed with seven different diets individually; fish was fed with reducing fishmeal levels (10, 20 or 50%) by either spinach

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leaf powder (SLP) or a chloroplast rich fraction (CRF) prepared by an established method to recover chloroplasts. Both SLP and CRF had a positive impact on growth, taste response, whole fishFA composition, and carotenoid profile. Fish fed with CRF diets showed significantly (P≤0.05) greater α-linolenic (C18:3 n-3) and hexadecatrienoic (C16:3) acids content than those of SLP and the control. Hexadecanoic acid (C16:3) is a unique FA in the galactolipids of the chloroplast; its presence in zebrafish tissues proves that zebrafish digest and absorb chloroplast galactolipids. Lutein profile of eggs produced by zebrafish fed with CRF diet was significantly (P≤0.05) higher than that of SLP and control. Alterations in egg colour were also noted, warranting further investigations of diet impacts on fish fecundity, embryo fertility, hatch rate and larval survival.

1. Introduction

Fish and its products can be consumed in various forms and are a good source of proteins, fats, vitamins and minerals (1). The aquaculture industry has grown in recent years to provide approximately 54% of world fish and seafood in 2016 (2), with e 70% of fish produced using aquaculture feeds in 2012 (3). Many of these feeds include fishmeal, as a source of high quality protein, and fish oil, which is high in unsaturated FAs, (4) to provide the necessary nutritional profiles for target species. Fishmeal and fish oil are produced from capture fish and fish by-products (3, 5), therefore the availability of both is affected by catch rates and raw material availability. As world aquaculture fish production has increased (from 39 million tonnes in 2011 – 54 million tonnes in 2016 (2), this puts extraordinary pressure on the supplies of fishmeal and fish oil. There is consequently a need to identify cheaper and more sustainable alternatives to both protein and oil materials, at least partially.

Zebrafish (*Danio rerio*) are now established as an excellent model organism for study in areas such as developmental biology, toxicology, genetics and medicine (6-9). They have also been used as model for studying lipid metabolism under the microscope (10).

Zebrafish share a high degree of amino acid sequence and functionality with mammals including humans. They also possess characteristics, including simple husbandry, gregarious behaviour, small adult size, rapid growth, development and maturation,

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high fecundity and productivity, which make them an ideal model species for pilot feed studies on novel and difficult to source or synthesis ingredients (11, 12).

SLP, and CRF were investigated as dietary ingredients in zebrafish to determine whether they could partially substitute for fishmeal fish feed. Chloroplasts are a type of semi-autonomous organelle found in plant leaves but also in other organisms like algae. They are responsible for photosynthesis, the process where sunlight energy is converted into chemical energy. It is in chloroplasts where valuable macro and micro-nutrients including protein (~50-60%), lipid (35-40%), and lipid soluble pigments such as chlorophylls, carotenoids (e.g. provitamin A and lutein) and tocopherols/vitamin E (5-10%) are synthesised (13-15).

Use of CRF as an ingredient facilitates the use of plants that are not conventionally consumed by humans or fish in feed manufacture adding valuable nutrients at the same time. The nutrient profile of CRF from green plant species shows it is rich in proteins, lipids, essential FAs, vitamins and minerals, which could be used as potential functional ingredients in human or animal food formulations (16, 17).

The major FA (>60%) in chloroplasts is the omega-3 FA known as α-linolenic acid, mainly in the form of galactolipids. Although ALA can be provided to fish by various plant sources, like vegetable oils, the most abundant source of ALA in the biosphere is galactolipids that make up the majority of the thylakoid membranes of chloroplasts. In our study the increase in fish-body ALA content can be attributed to the digestion and update of ALA from the galactolipids in the SLP and CRF material.

A portion of this FA is converted by zebrafish into long chain PUFAs, found in fish lipids (18). PUFAs are important dietary sources for eicosapentaenoic (EPA, 20:5ω-3), and docosahexaenoic (DHA, 22:6ω-3) acids, which are believed to have health benefits, including promotion of proper growth of children and, prevention of cardiovascular diseases and cancer (19). Consumption of fish oils also contributes to energy supplies and assists in the proper absorption of fat soluble vitamins namely A, D, E, and K through digestion (20).

2. This study investigated the effectiveness of partial inclusion of SLP and CRF in fish meal to

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enhance fish growth and uptake of the signature compounds (certain micro-nutrients i.e. carotenoids e.g., lutein and β-carotene and FAs e.g. alpha-Linolenic acid and Hexadecatrienoic acid) used as indicative markers for ingredient digestibility. To the best of our knowledge, it is the first time that a galactolipid specific FA, particularly of monoglactosyldiglycerides (MGDG), has been shown to be stored in animal tissue. In addition, as zebrafish is a good model for biological studies, successful ingredient digestibility by zebrafish is used as a good indicator for potential successful diet digestibility by human. Spinach and its CRF was used as a model to represent green plant materials, because of its availability all year round.

3. Material and Methods

3.1. Diets

A control diet was formulated to meet the nutritional requirements of zebrafish using standard aquaculture ingredients. Experimental diets were designed based on a control diet with 10%, 20% and 50% reduction in fishmeal content. The SLP and CRF materials were incorporated into each experimental diet at maximum levels determined by their nutritional profiles, while maintaining isonitrogenous and isocalorific characteristics with the control as such the diets are labelled: Control, SLP10, SLP20, SLP50, CRF10, CRF20 and CRF50, see table 1 (A).

Complete diets were formulated in house at the University of Liverpool, to meet or exceed the nutritional requirements of the experimental species, see table 1 (B) for the nutritional profile of each diet. SLP and CRF recovery was based on a previous study (16), carried out in our laboratory, and their relevant compositional analysis to this study is depicted in Table 2.

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Table 1 Experimental diet compositions showing ingredient inclusion rates (A), and nutritional profile of experimental diets (B)

Diet (% wt)								**A**
		Control	CRF10	CRF20	CRF50	SLP10	SLP20	SLP50
Fish Meal		38.74	34.76	29.96	19.77	35.59	30.09	19.10
Rapeseed Oil		4.37	-	-	-	-	-	-
Vitamin premix		0.31	0.33	0.32	0.33	0.32	0.32	0.32
Mineral premix		0.42	0.43	0.43	0.44	0.43	0.43	0.42
Wheat Gluten		22.91	18.95	22.50	31.33	19.85	23.38	30.95
Corn Starch		31.82	28.04	26.12	25.85	29.97	28.75	26.12
Binder (CMC powder)		0.52	0.54	0.53	0.55	0.54	0.54	0.53
CRF		-	15.21	18.19	19.20	-	-	-
SLP		-	-	-	-	11.77	14.65	20.15
Arginine		-	0.28	0.34	0.55	0.22	0.32	0.51
Leucine		-	0.35	0.35	0.37	0.25	0.29	0.33
Lysine		0.90	1.10	1.26	1.61	1.04	1.21	1.55
Nutrient								**B**
Crude Protein	% DM	46.56	46.91	47.77	48.43	45.94	46.04	46.58
Crude Lipid	% DM	11.30	11.72	12.43	12.09	8.56	8.64	8.79
Carbohydrate	% DM	35.06	33.38	32.04	32.14	35.99	35.58	34.53
Crude Ash	% DM	7.94	7.79	7.01	5.15	9.53	9.04	8.01
Crude Fibre	% DM	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Oleic acid	mg/g DM	30.02	3.7	4.34	5.25	2.85	3.35	4.38
LA	mg/g DM	20.20	13.02	15.16	19.23	12.90	15.07	14.76
ALA	mg/g DM	12.63	41.29	46.91	47.00	21.08	23.22	27.20
EPA	mg/g DM	3.29	2.95	2.55	1.68	3.03	2.56	1.62
DHA	mg/g DM	9.60	8.62	7.43	4.90	8.82	7.46	4.72
*Lutein	(µg/g) DM	0.0	520	620	650	100	130	180
Gross Energy MJ/kg DM		21.28	19.24	19.07	18.93	19.36	19.09	18.62

CRF: chloroplast rich fraction; SLP: spinach leaf powder; wt %: weight percent; CMC: carboxymethyl cellulose; LA: Linoleic acid; ALA: alpha-Linolenic acid; ARA: arachidonic acid; Amino acid profiles were estimated for each diet during formulation, the control diet was supplemented with crystalline amino acid lysine to boost levels close to that required by Zebrafish (21). Further supplementation of each experimental diet took place using arginine, leucine and lysine, where required to achieve amino acid profiles which remained consistent with the control diet. * Lutein levels in diets predicted from CRF and SLP material only.

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Table 2 Compositional analysis of CRF and SLP (mg g^{-1} D.M)

	Protein	Lipid	*Car.	Lutein	β-car.		
CRF	426±1	369±13	14.7±4.6	3.41±0.12	3.36±0.06		
SLP	353±45	193±16	2.2±0.2	0.87±0.09	0.76±0.08		
			FA				
	C16:0	C16:1	C16:3n-3	C18:0	C18:1n9c	C18:2n-6c	C18:3n-3
CRF	10.5±2	4.2±1	13.8±2	0.7±0.1	3.0±0.6	5.9±1	57.9±7.8
SLP	6.3±0.3	1.2±0.9	5.4±0.3	0.6±0.0	1.9±0.1	6.8±0.3	32.6±1.4

*Car: Total carotenoids (xanthophylls +carotenes); β-car: β-carotene; FA: fatty acid. Data in this table is adapted from (16). **C16:0**, Palmitic acid; **C16:1**, Palmitoleic acid; **C16:3 n-3**, Hexadecatrienoic acid; **C18:0**, Stearic acid; **C18:1n-9c**, Oleic acid; **C18:2 n-6c**, Linoleic acid (LA); **C18:3n-3,** α-Linolenic acid (ALA)

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3.2. Preparation

All ingredients required were thoroughly mixed using a Hobart food mixer andwater was added until the mixture achieved dough-like consistency. Macro nutrients were added first, followed by micro (added while mixing took place), and liquids were added last. The dough was spread out on trays and dried for 24 h at 50°C using a nine shelf Parallexx Excalibur food dehydrator. Once dry, the diet was crushed and sieved through a series of sieves with apertures of 425 µm and 850 µm. The desired pellet size (size?) fell between the two. All diets were refrigerated at +4°C for the duration of the study.

3.3. Subjects and Husbandry

All fish were bred in-house at The Institute of Integrative Biology aquarium facility, the University of Liverpool. The first trial took place over an eight week time period and involved 525 juvenile *D. rerio* (AB wild type strain). The fish were approximately two months old at trial start. They weighed on average 0.041 ± 0.008 (STDEV). Fish were housed in groups of 15 individuals in 35 identical 1.5 L zebrafish tanks, made by Aquatic Habitats, each measuring 25 cm x 7 cm x 15.5 cm. The tanks were connected to a central system maintained by a sump filtration system and 20% weekly water changes. Due to the small size of the fish, tanks were fitted with a 400 µm fry mesh baffle; cleaning was conducted weekly during the weighing of the fish to prevent further disturbances. Water quality was controlled within the following parameters, Ammonia (NH_4); 0 mg L^{-1}, Nitrite (NO_2); 0 mg L^{-1}, Nitrate (NO_3); <50 mg L^{-1} and pH; 7.0. Fish were maintained at 28±1°C and exposed to a 12/12 hour light cycle.

For the second trial, 84 mature *D. rerio* (Casper strain) were used. The fish were approximately seven months old at trial start. Fish were housed in groups of six individuals, either all male or all female, in 14 identical tanks, the same 1.5 L tanks as above using the same baffles. The tanks were maintained on the same system as above with the

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same water quality parameters and environmental conditions. Cleaning of tanks was carried while fish were housed in breeding tanks.

3.4. Experimental Procedure

3.4.1. Weighing Fish

Fish were weighed by tank (15 fish) weekly, to measure growtht. A separate 1.5 L tank was used, filled with one cm of system water, placed on a Fisher scientific SG-602 balance and tared. All 15 fish were caught in a small net, lifted from the housing tank, excess water was removed by blotting the net and then the fish were transferred to the tank on the balance. The weight was recorded for each tank. While the fish were situated in the weighing tank, the housing tank and mesh baffle were cleaned before returning the fish.

3.4.2. Feeding and Sampling

Fish were hand fed 4% body weight using Sarstedt 1.5mL micro tubes containing pre-measured feed amounts, measured accurately to 3 decimal places using a five point decimal place Kern 770-60 laboratory weigh balance (linearity ±0.03mg) per day calculated weekly. Each experimental diet was fed to five replicate tanks, 75 fish in total. At the end of the trial the fish were euthanized using a Home Office approved Schedule 1 method; concussion followed by pithing of the brain to confirm death. Fish were then weighed and measured individually. Fish from all five tanks on the same diet were collated together, frozen at -20°C transported on dry ice to the Division of Food Science, the University of Nottingham and stored at -80°C. From the measurements collected, percentage growth, feed conversion ratio (FCR), and specific growth rate (SGR) were calculated as diet efficiency and performance indicators. FCR indicates the efficiency at which feed is converted into animal biomass, calculated as follows:

$$\text{FCR} = \frac{\text{Total feed intake (kg)}}{\text{Net aquacultural production (kg)}}$$

Total Feed Intake (TFI) = total feed given – waste output.
Net production = Mass at end of study period – mass at start of study period. (22)

SGR demonstrates the growth achieved per day during a time period of which subjects are fed the test diet, calculated as follows:

$$\text{SGR (\%)} = 100 \times (\text{Ln}W_2 - \text{Ln}W_1) \times (t_2 - t_1)^{-1}$$

Where:
Ln = natural log
W_1 = Initial weight
W_2 = Final weight

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t_1 = Starting time point (day one)
t_2 = End time point (final day number) (23)

3.5. Diet Palatability and Acceptance

Feeding observations were carried out weekly to assess fish response and feed acceptance. The amount (%) of feed (4% body weight of all fish in the tank) that remained at 20 second time intervals during the five minute period post feed introduction was estimated to the nearest 10%. Observers were trained to recognise the amount of feed remaining in a tank.

3.6. Zebrafish Spawning

Each of the seven diets was fed to two tanks, one male and one female tank, six fish per tank. Feed was given daily *ad libitum* until satiation. After three weeks of feeding the fish were bred by mixing males and females together (1:1 male: female ratio) one evening in medium (3 L) Aquatic Habitat zebrafish tanks, measuring 11.5 cm x 25 cm x 15 cm. A mesh net with a 2 mm aperture was placed in the tank to create a false raised base with a gradient to prevent cannibalism of the eggs. As soon as the lights came on the next day the flow to the tank was switched off and the net was raised slightly to stimulate spawning. After two hours the fish were removed and returned to all male or all female housing. Eggs were collected, transferred to sample jars then snap frozen with liquid nitrogen; samples were stored at -80°C. Spawning was repeated every three days until sufficient eggs had been collected for the required sample size. Fish were then euthanized using a Home Office approved Schedule 1 method. Fish were also stored at -80°C until further analyses.

3.7. Ethical Issues

The work carried out here was done so under the Establishment Licence for the University of Liverpool (X70548BEB). Therefore, meeting all the standards required under the Animals (Scientific Procedures) Act 1986. The work was thoroughly reviewed, ethical approval was granted (ref number AWC0082) via the University's AWERB (Animal Welfare and Ethical Review Body), and the work was deemed below threshold for regulation. Throughout the trial fish welfare was independently monitored by animal and care welfare officer (NACWO) and was not compromised during this study.

3.8. Post-trial Preparation and Chemical Analysis of Zebrafish

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Zebrafish and eggs were freeze dried for one week (Edwards Freeze Dryer, Super Modulyo). During the freeze drying process, samples were protected from light exposure. Samples were then ground to homogeneous powder using mortar and pestle under dim light and with liquid nitrogen.

3.8.1. Lipid Extraction

Lipids were extracted from the freeze dried materials (control, as well as, CRFs or SLP fed zebrafish samples) using a modified Folch, Lees (24) technique. A ratio of chloroform: methanol (2:1) was firstly prepared, and 6 mL was added to 0.5 g (1.2 mL solvent to 0.1 g of eggs) of freeze dried sample and vortexed for 1 min. To this 2.5 mL (0.5 mL for the eggs) of a 0.9% sodium chloride solution was added, and the mixture was vortexed (1 min) before being centrifuged, using a Thermo Jouan CR3i multifunction centrifuge (1300 RCF for 10 min at 4°C) to separate into three layers. The lower phase, containing the lipids and chloroform, was transferred to a clean vessel. A further 6 mL of chloroform: methanol (2:1) was added to the remaining two phases which were then vortexed and centrifuged to the same conditions as above. The lipid phase was removed and pooled with the original lipid. The pooled lipids were centrifuged (same conditions as above) to separate the lipids from any residual compounds content, they were then filtered (0.45 µm PTFE syringe filter) before being dried under a flow of nitrogen. The combined lipid extracts were then weighed, quantified gravimetrically and stored in -80°C until further analysis.

3.8.2. Fatty Acid Composition

The FA content of the zebrafish was determined by esterification of total lipid extracts, obtained from section 2.8.1 to fatty acid methyl esters (FAMEs) and analysed using gas chromatography-mass spectrometry (GC-MS). The dried lipid extracts were dissolved in 2 mL of chloroform; 100 µL (10 mg mL^{-1}) of methyl pentadecanoate (internal standard) was then added to 1 mL of the lipid extract in chloroform. Esterification was achieved through the addition of 200 µL of trimethylsulfonium hydroxide. The solution was then left, to ensure complete conversion, for 10 min before injection onto the GC-MS (Thermo Scientific, DSQII). GC-MS conditions were based on Bahrami, Yonekura (25). Briefly, samples were injected at a volume of 10 µL onto a Phenomenex Zebron ZB-FFAP (30 m ×

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0.22 mm) column using a vaporising injector with a split flow of 50 mL min^{-1} of the carrier gas (helium). The oven temperature was maintained at 120 °C for 1 min, and then increased to 250 °C at a ramp of 5° min^{-1}. The final temperature of 250 °C was held for 2 min. Detection was conducted using a mass spectrometer and identification of individual FA was achieved using a mass spectrum library and its comparison of retention times to FAME standards. The percentage content of each FA was calculated and the concentrations (mg mL^{-1}) were determined using the response factor of the internal standard.

3.8.3. Zebrafish Retinol Analysis

The analysis and detection of all trans retinol in the whole zebrafish for test trials and controls was based on Li, Tyndale (26) with slight modifications. Briefly, nitrogen-dried lipids obtained from section 2.8.1 were redissolved in 1 mL acetone and vortexed (1 min). Extracts were then vigorously mixed with an equivalent volume of methyl-tetra-butyl ether (MtBE) and 0.5 mL distilled water. The aqueous phase was extracted further three times using the MtBE. The combined MtBE phases were evaporated to dryness under a gentle stream of nitrogen, suspended with 0.5 mL MtBE, vortexed and passed through a PTFE membrane filter (0.45 µm). The extracts were sealed under nitrogen in brown glass vials and stored in the dark at -80ºC. Retinol analysis was carried out using an Agilent 1100 HPLC equipped with a diode array UV detector. Separation was achieved with a Gemini-NX C18 column (250 mm X 4.6 mm, 5 µm) coupled with a C18 guard column (Phenomenex, UK). Absorption spectra were recorded at 325 nm for retinol. The mobile phase consisted of two components: (A) methanol and (B) MtBE. The solvents program was initially 90% A from 0-12 min, followed by linear gradients of 90-60% A from 12-13 min, maintained until 22 min followed by linear gradient back to 90% A initial conditions until end of run at 30 min. The flow rate was 0.8 mL min^{-1}. Column conditions were maintained at ambient temperature. The injection volume was 10 µL and retinol quantified using external standard.

3.8.4. Analysis of Carotenoids in Whole Zebrafish and Eggs

Carotenoids were extracted as section 2.8.3, but the detection program of the HPLC was different and was based on Garner, Neff (27). Briefly, two mobile

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phases: (A) acetonitrile, methanol, 0.1M Tris-HCl pH=8 (7:1:1) and (B) methanol, hexane (4:1) were prepared. Separation began by injection of 10 µL of sample, followed by 4 min of 100% solvent A, 2.5 min linear gradient to 100% solvent A and 16 min at 100 solvent A to re-equilibrate the column before loading the next sample. The flow rate was 1 mL min^{-1} with all solvents used being HPLC grade and the wavelength at 480 and 450 nm.

3.9. Statistical Analysis

Experiments were performed in triplicate. i.e. three separate samples for each experiment (except for lipid extraction, where three samples of pooled freeze-dried fish bodies subjected to the same diet was used) and the statistical analysis was carried out using the Minitab V. 17 statistical package (Minitab Inc., PA, USA) using post-hoc analysis of variance (ANOVA) and according to Fishers' test with statistical significance at $P \leq 0.05$ and $P \leq 0.001$. It should be noted here that during the growth and performance trial, five replicate tanks were used for each of the seven experimental feeds, including the control.

4. Results and Discussions

4.1. Growth, Performance and Palatability

Both the CRF and SLP materials had a positive impact on growth (Fig.1). No significant differences ($P \geq 0.05$) were found in growth between the control diet and any of the experimental diets, except for the CRF10 and SLP10 diets, which showed significantly ($P \leq 0.05$) increased growth compared with the control (Fig.1). The only significant differences between the control diet and any experimental diet for FCR was a decrease in both CRF10 and SLP10, showing improved feed conversion. There were no significant differences found between the control diet and any experimental diet for SGR. The SLP10 and CRF10 diets also showed significantly improved growth, FCR and SGR when compared to the SLP50 diet, as indicated on fig.1. These results show that use of SLP and CRF materials successfully aided fishmeal reduction up to a level of 50%. However, comparisons between experimental diets also indicate reduced feed performance and efficiency in *D. rerio* with increasing fishmeal reduction when these materials are used, with the SLP50 diet performing significantly poorer in growth, FCR and SGR than

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both the 10% reduction diets. It is not clear at this stage why increased SLP resulted in lower growth rate.

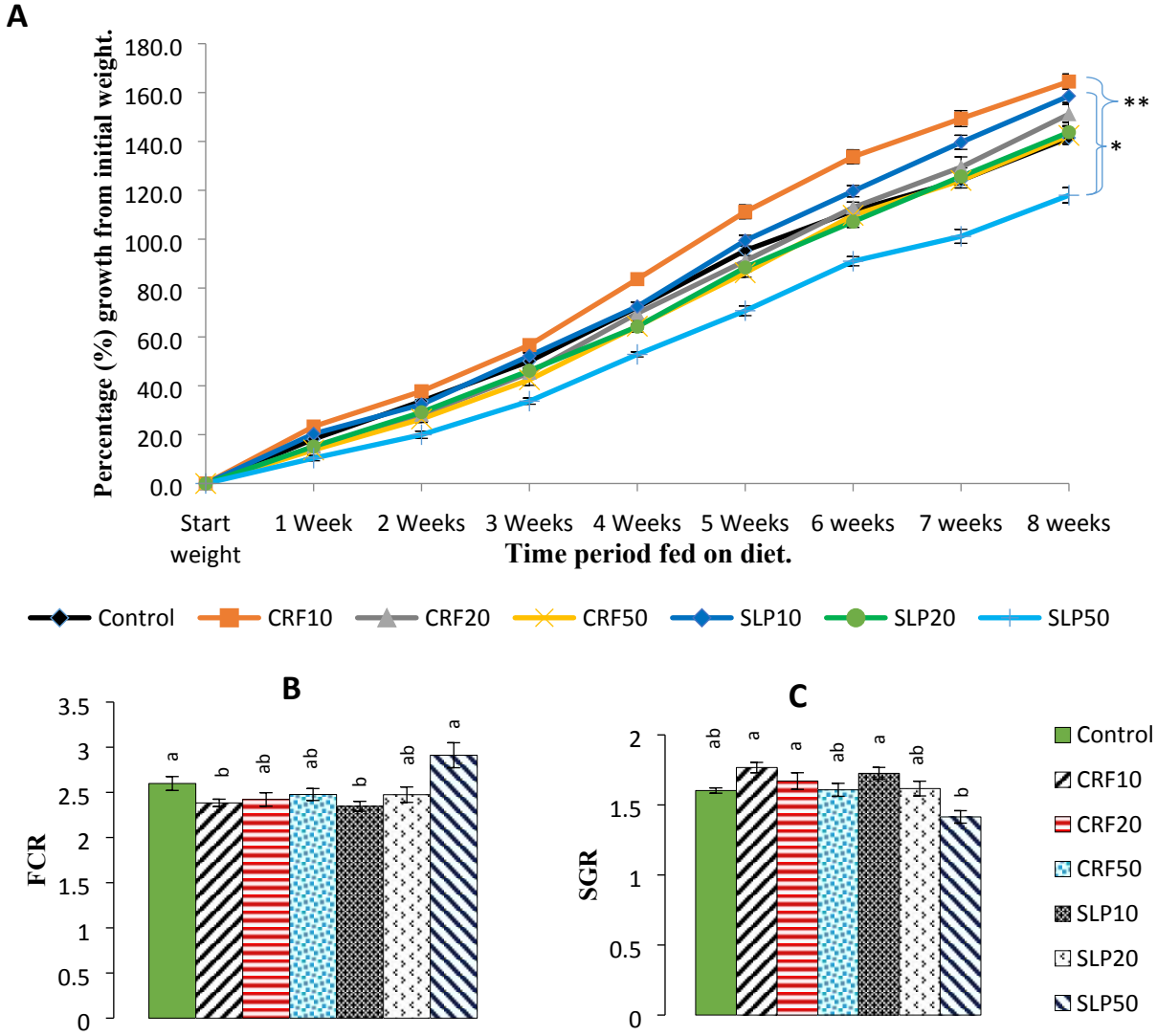

Figure 1 Mean (±se) values for growth and performance indicators for fish fed experimental diets. A) Percentage growth from initial weight. Significance indicated as follows: **P<0.01,*P<0.05. B) Feed conversion ratio (FCR). C) Specific growth rate (SGR). Within each figure section, diets that do not share a letter are significantly different (P≤0.05). CRF: chloroplast rich fraction; SLP: spinach leaf powder.

Although comparison of CRF and SLP digestibility was not our target at this stage, we anticipated that the digestion of the CRF material by zebrafish would

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be more efficient compared with the SLP material, due to the liberation of chloroplasts from the cell wall matrix in the CRF material. However, cell wall damage caused by the method of preparation to make the SLP (16) probably made SLP as easily digestible as the CRF for zebrafish (Fig. 2). A recent study on the impact of protein and fibre content on gut structure and function of zebrafish was conducted; and it was found that fish consuming a herbivorous diet (containing high cellulose) had similar body growth (P≥0.05) as those consuming an omnivorous diet (zebrafish are omnivorous in nature). However, no cellulose-degrading enzymes (cellobiohydrolase and β-glicosidase) could be detected in the guts of the zebrafish, used in the study (28).

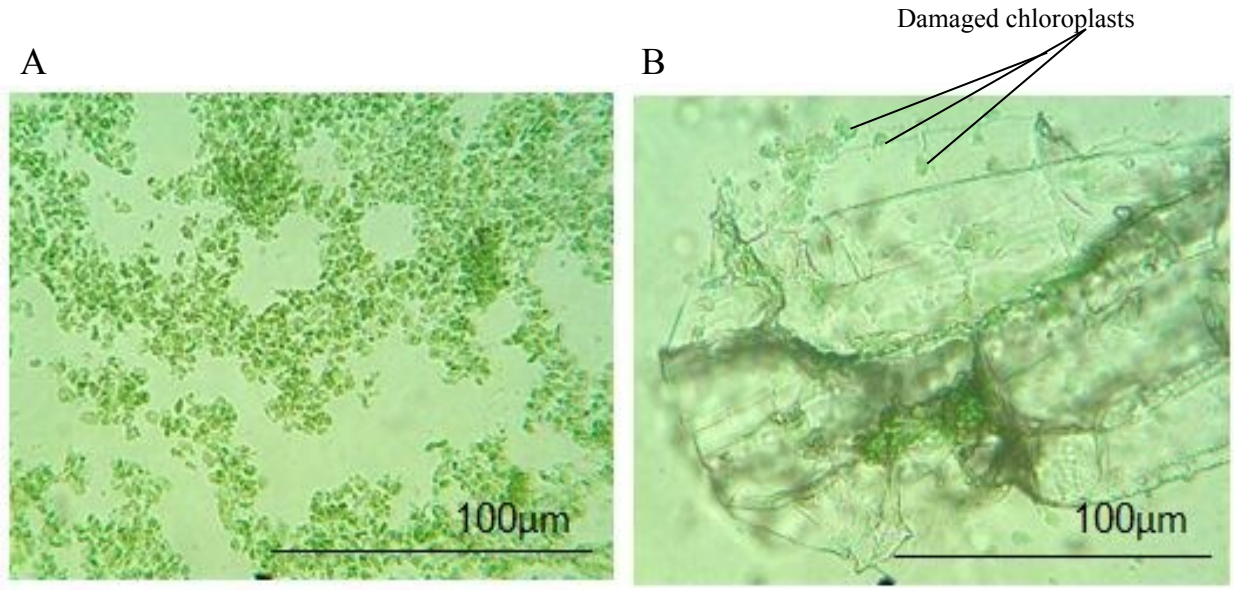

Figure 2 Representative microstructure of intact CRF (A) and SLP with partially damaged CRF dots (B). Optical microscopy with differential interference contrast microscopy (DIC) was used (Leitz Diaplan Microscope). A drop of each sample (initially dissolved with water) was placed on a glass slide, topped with a cover slip and allowed to dry before being placed under the microscope. All images were taken using a 100X objective lens. A digital camera was attached to the microscope to capture images of the samples. The scales of the images were calibrated against a glass mounted graticule (1 mm, 0.01 mm division from Graticules Ltd, Tonbridge).

Assessment of diet consumption rate indicates that inclusion of both the CRF and SLP materials has a positive impact on diet taste or appearance. Fig.3 shows that all experimental diets were consumed at

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an increased rate, showing significantly improved palatability compared to the control.

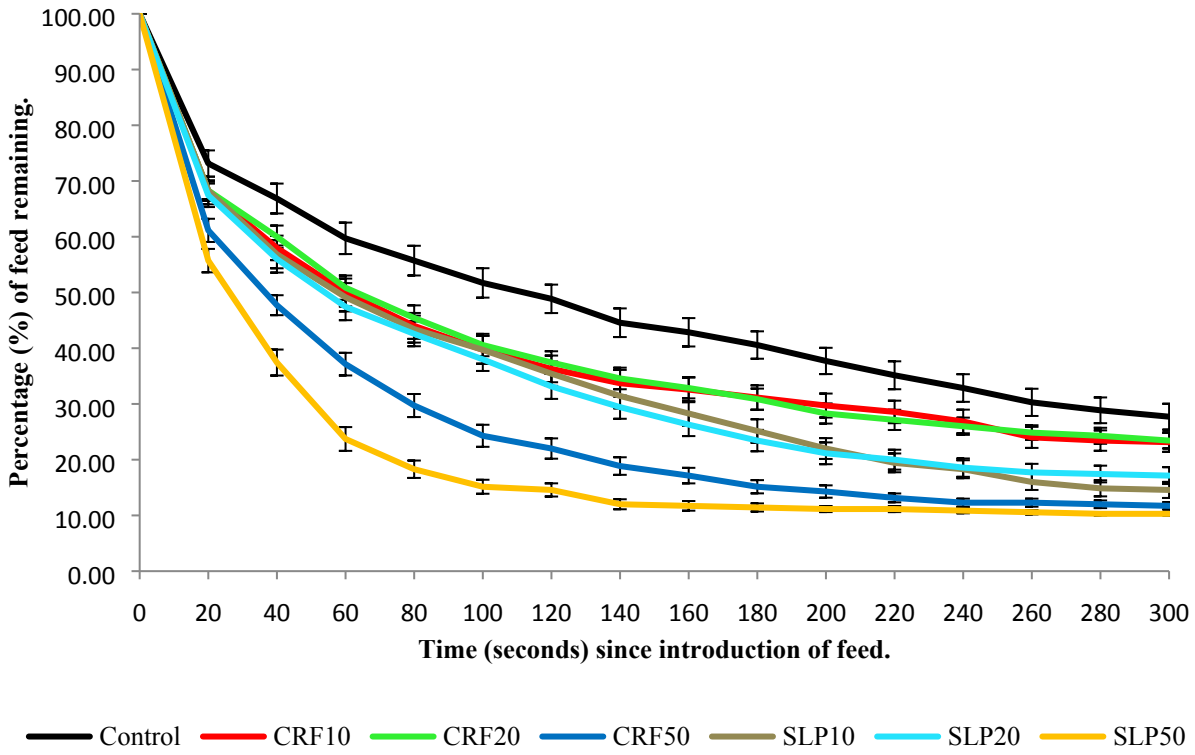

Figure 3 Mean (±SE) percentage of diet (a 4% body weight ration of all fish in tank) remaining (to the nearest 10%) at 20 second intervals for five minutes post introduction of feed. Recordings for each tank stopped once remaining diet volume reached 10%. CRF: CRF: chloroplast rich fraction; SLP: spinach leaf powder.

4.2. Fatty Acid Composition

The FA composition of the trial zebrafish is presented in Table (3). FA composition of fish reflects the FA composition of the diet. Oleic and linoleic acids (LA) were seen in higher proportions ($P \leq 0.05$) in the fish fed the control (fish meal) diet compared to the fish fed the CRF and SLP feeds. In contrast, major FAs in CRFs and SLP such as palmitic acid (C16:0), hexadecatrienoic acid (C16:3) and α-linolenic acid (C18:n3; ALA) were all significantly ($P \leq 0.05$) higher in the zebrafish fed the experimental diets (mainly in CRF but also in SLP test diets) compared to the controls. Most of these FAs occur in chloroplast thylakoids in the form of

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galactoplids, mainly monoglactosyldiglycerides (MGDG) and diglactosyldiglycerides (DGDG). The metabolism of chloroplast glactolipid-derived α-linolenic acid to EPA and DHA is apparent. Given that DHA concentration declines as fishmeal in the diet declines (Table 1), and that DHA remains relatively stable in the body of the fish across all the diets (Table 3), suggests either that chloroplast α-linolenic acid is converted, not only to EPA but to DHA, or that the fish feed contains enough DHA, even in the CRF 50 and SLP 50 diets, to take up a required level of this FA.

Zebrafish expresses similar functional fatty acyl desaturates, Δ6 and Δ5 to those of human, which are essential enzymes for the biosynthesis of PUFAs (29). In humans and probably zebrafish (expressing Δ6 and Δ5), 8% and 4% of the essential FAs, for example, α-linolenic acid is converted into EPA and DHA, respectively (30). However, conversion of those FAs to DHA is often limited. A recent study on human subjects demonstrated that a high-ALA diet of 14.0 g day^{-1} significantly increased ALA and EPA concentrations in red blood cells but not for DHA (31).

Conversion of essential FAs such as LA and ALA to PUFAs is influenced by their balance in the diets (32) as LA and ALA compete for the same elongase and desaturase enzymes in the synthesis of EPA and DHA (33). The conversion of ALA to EPA and DHA is disrupted and reduced when the ratio of LA to ALA is high (34). Harnack, Andersen (35), reported that the rate of EPA and DHA formation was greatly increased when the ratio between LA and ALA was about 1:1.

Owing to their expression of two functional fatty acyl desaturases (Δ6 and Δ5), zebrafish are among a number of animal species with the ability to convert C18 FAs (via elongation and desaturation on both n-3 and n-6 FA) into PUFAs. In freshwater fish the dietary linoleic acid in the phospholipid fraction is elongated and desaturated and converted to Arachidonic acid (ARA) and osbond acid (Docosapentaenoic acid, C22:5 n-6); whereas, linolenic acid is elongated and desaturated to DHA, (36)'

The ratio of ALA to EPA (4:1) concentration (mg g^{-1}) for CRF diets (by average) in zebrafish body post-feed analysis was calculated from Table 3.

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Values were in line with that of zebrafish, fed a commercial meal i.e. 4:1 of ALA to EPA for the non-transgenic sample of a previous study by Yoshizaki, Kiron (37).

Table 3 FA composition of whole body zebrafish fed with various test diets

FA (mg g^{-1} D.M)	Control	CRF 10	CRF 20	CRF 50	SLP 10	SLP20	SLP50
C14:0	1.9±0.0^{B}	2.2±0.0^{A}	2.1±0.0^{A}	1.8±0.0^{BC}	2.1±0.1^{A}	2.1±0.0^{A}	1.7±0.0^{C}
C16:0	21.5±0.3^{E}	24.9±1.8^{CD}	27.2±0.3^{AB}	28.9±0.1^{A}	23.2±1^{DC}	26.3±0.4^{BC}	26.2±0.9^{BC}
C16:1n-7	2.4±0.1^{CD}	2.7±0.2^{AB}	2.8±0.0^{AB}	2.6±0.0^{BC}	2.7±0.1^{ABC}	2.9±0.0^{A}	2.2±0.2^{D}
C16:3 n-3	0.03±0.0^{F}	0.9±0.0^{B}	1.1±0.0^{A}	1.1±0.0^{A}	0.3±0.0^{E}	0.4±0.0^{D}	0.4±0.0^{C}
C18:0	3.0±0.0^{B}	4.3±0.6^{A}	4.6±0.1^{A}	4.4±0.2^{A}	4.7±0.3^{A}	4.8±0.2^{A}	4.7±0.3^{A}
C18:1 n-9c	20.2±1.4^{A}	18.4±1.7^{AB}	17.8±0.2^{BC}	19.7±0.4^{AB}	15.7±0.7^{C}	18.0±0.0^{AB}	17.6±0.8^{BC}
C18:2 n- 6c	15.0±0.5^{A}	9.9±1.3^{D}	11.8±0.0^{C}	13.7±0.0^{AB}	11.4±0.7^{CD}	12.9±0.0^{BC}	12.8±0.8^{BC}
C18:3 n-3	3.0±0.0^{E}	5.7±0.9^{B}	7.5±0.1^{A}	7.3±0.0^{A}	3.2±0.1^{DE}	3.9±0.0^{CD}	4.1±0.2^{C}
C20:4 n- 6	0.5±0.0^{A}	0.55±0.0^{A}	0.5±0.0^{A}	0.5±0.0^{A}	0.5±0.0^{A}	0.5±0.0^{A}	0.6±0.0^{A}
C20:5 n- 3 (EPA)	1.5±0.1^{BC}	1.9±0.2^{A}	1.8±0.0^{A}	1.4±0.0^{C}	1.8±0.0^{A}	1.7±0.0A^{B}	1.4±0.0^{C}
C22:6 n- 3 (DHA)	6.0±0.1^{AB}	6.6±1.4^{AB}	7.3±0.0^{A}	6.2±0.1^{AB}	6.8±0.2^{AB}	6.7±0.0^{AB}	5.6±0.3^{B}
∑SFA	26.40	31.40	33.90	35.10	30.00	33.20	32.60
∑MUFA	22.60	21.10	20.60	22.30	18.40	20.90	19.80
∑PUFA	26.03	25.55	30.00	30.20	24.00	26.10	24.90
∑n-6 FA	15.50	10.45	12.30	14.20	11.90	13.40	13.40
∑n-3 FA	10.50	15.10	17.70	16.00	12.10	12.70	11.40

DM, dry matter; **C14:0**, Myristic acid; **C16:0**, Palmitic acid; **C16:1**, Palmitoleic acid; **C16:3 n-3**, Hexadecatrienoic acid; **C18:0**, Stearic acid; **C18:1n-9c**, Oleic acid; **C18:2 n-6c**, Linoleic acid (LA); **C18:3 n-3**, α-Linolenic acid (ALA); **C20:4 n-6**; Arachidonic acid; **C20:5 n-3 (ARA)**, Eicosapentaenoic acid (EPA); **C22:6 n-3**, Docosahexaenoic acid (DHA); **FA**: fatty acid; **SFA**: saturated FA; **MUFA**: mono unsaturated FA; **PUFAs**: poly unsaturated FAs. **CRF**: chloroplast rich fraction; **SLP**: Spinach leaf powder. Means that do not share a letter within each row are significantly different.

FAs such as C18:3 n-3 and C16:3n-3 are predominantly found in green leaves and their chloroplast membranes; to the extent that they are used for the categorization of certain green plants as being C18:3n-3 or C16:3n-3 plants (38). The lower ($P \leq 0.05$) profile of C18:3 n-3 and very low concentration of C16:3 n-3 shown by the control compared to the experimental feeds may suggest use of C16:3 n-3 as an indicative marker of feed transition into the fish body. This indicates that the diets, notably CRF, were digested and the nutrients were absorbed from the feed so contributing to the final FA accumulation in the fish body. Similarly, ALA was used as an indicator of chloroplast FA, and its uptake into the zebrafish is clear on comparing fish body ALA composition in the control fish diet with that of fish fed the CRF or SLP supplemented

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diet. In both cases this increase in ALA appears to be dose dependent.

4.3. Carotenoid Content in Zebrafish (Whole)

Lutein and beta-carotene are the major carotenoids in CRF and SLP whilst astaxanthin is the prominent carotenoid in many fish species (mainly marine fish) as a result of conversion of dietary carotenoids (from aquafeeds or natural diets e.g. marine algae) into astaxanthin in fish *in vivo*. Beta-carotene was not detected in test samples or the control; and quite low concentrations of astaxanthin (data not given) were detected only for CRF10 and CRF20 diets but not for the rest of the zebrafish treatments or the control. Lutein was, however, the major carotenoid found in the whole body of zebrafish; and its concentrations varied depending on the type of the feed and the concentration. Feeds from CRFs initially (in the raw materials) containing higher carotenoids than the SLP, showed also higher lutein content in the zebrafish (table 4). It should be noted here that in terms of chloroplast content, CRF10 was approximately equivalent to SLP50 in chloroplast content.

Carotenoids are pigments found exclusively in photosynthetic organisms such as, plants, algae, few fungi and certain bacteria (39). They constitute a group of over 700 structures responsible for the yellow, red and orange colours of many fruits, vegetables and flowers (39). Carotenoids belong to the C40- based isoprenoid family termed tetraterpenes (40, 41). Categorically, carotenoids are of two main groups: xanthophylls (e.g. lutein, zeaxanthin, neoxanthin, violaxanthin and β-cryptoxanthin) with oxygen in their carbon chain and carotenes (e.g. α-carotene, β-carotene and lycopene) which are purely hydrocarbons and well distinguished for being devoid of oxygen. Where green vegetables contain high amounts of both oxygenated and non-oxygenated carotenoids, lycopene, an oxygenated carotenoid is present in ripe tomato fruit as a lipophilic red pigment and carrots are orange in colour as a result of β-carotene. Similarly, whilst the red colour of pepper is due to capsanthin, the pink red coloration of crustaceans is accounted for by astaxanthin.

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The manner in which carotenoids are metabolized is greatly dependent on the fish species. Being fat-soluble, dietary carotenoids are assumed to be in micellar form in the intestine, together with bile salts, FAs, monoglycerides and other fat-soluble vitamins (42). Carotenoids are believed to be passively diffused into the intestinal lumen, and the uptake seems to be a slow process taking between 18 and 30 hours (43). It was reported that lutein was the major pigment in salmon and trout if the diet had contained plant carotenoids (44).

Table 4 Lutein and retinol concentrations in whole body of zebrafish and its eggs (mg kg^{-1} D.M)

Diet	Lutein (whole fish)	Lutein (egg)	Retinol (whole fish)
Control	0.1 ± 0.04^{D}	23.4 ± 2.4^{C}	0.4 ± 0.1^{C}
CRF10	3.8 ± 0.5^{BC}	53.5 ± 13^{AB}	1.7 ± 0.04^{B}
CRF20	5.2 ± 1.2^{A}	66.2 ± 19^{A}	1.5 ± 0.1^{B}
CRF50	4.6 ± 0.2^{AB}	60.5 ± 4.5^{A}	2.0 ± 0.8^{B}
SLP10	1.3 ± 0.2^{D}	33.9 ± 8^{BC}	1.8 ± 0.1^{B}
SLP20	3.5 ± 0.1^{BC}	32.4 ± 6^{BC}	3.5 ± 0.8^{A}
SLP50	2.7 ± 0.1^{C}	27.5 ± 6^{C}	2.4 ± 0.2^{B}

Means that do not share a letter within each column are significantly different; CRF: chloroplast rich fraction; SLP: whole leaf material.

In freshwater fish, β-carotene is converted into retinoic acid and in some cases into retinol, whilst lutein is converted into anhydrolutein, which, in turn, is cleaved to dehydroretinol (vitamin A_2), the common form of vitamin A found in most fresh water fish (45). From our data it seems likely that β-carotene was not detected in zebrafish because it is converted into retinol and retinoic acid.

4.4. Zebrafish Egg Carotenoids

It was noted that feed composition has a significant impact on fish egg colour (Fig.4). We explored this further using Casper strain zebrafish. The yellow colour in the eggs was thought to be due to accumulation of carotenoids, and that, as the concentration of the carotenoids in the test diet increased, the intensity of the yellow colours would also increase, viz. CRFs with increased carotenoid content per unit mass would yield more intense yellow colour compared with the SLP. However, the yellow colour did not correlate with the carotenoid concentration among CRFs. The eggs of CRF10 and CRF20 fed fish indicated noticeably more intense yellow colours than the CRF50 fed fish and more intense than all SLP enriched feeds. The control samples, were completely transparent; supporting our assumption that the yellow colour could be attributed to the carotenoid-rich samples (e.g. CRF).

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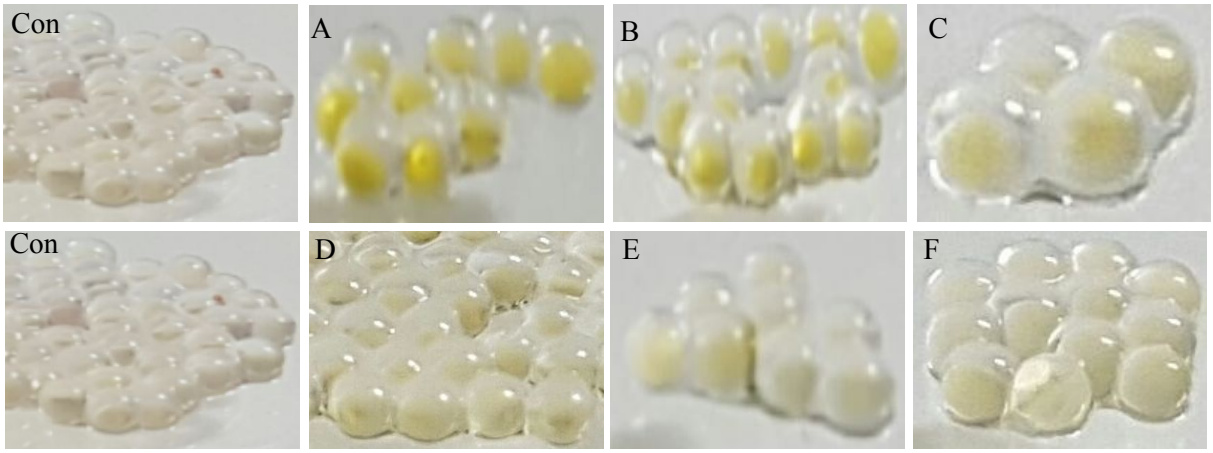

Figure 4 Egg colour variations depending on the zebrafish feed type; Con: control; A: CRF10; B: CRF20; C: CRF50; D: SLP10; E: SLP20, F: SLP. These images were taken with a SAMSUNG Galaxy S7.

The AB wild type zebrafish pigmentation consists of three cells: black melanophores, reflective iridiphores and yellow xanthophores (46). The Casper strain zebrafish only have yellow xanthophores, lacking both the black melanophores and reflective iridiphores resulting in a mostly transpareant fish (47). This enables the eggs to be seen within the females. The yellow colour pigmentation resulting from the diets accumulated differently in each sex. Yellow pigmented eggs can be seen within females, whereas in males the yellow pigmentation is dispersed throughout the whole body (Fig.5). Carotenoids (e.g. β-carotene and Lutein) are yellow/orange pigments, which are vital for healthy vision, providing protection from blue wavelengths of light (9). For the males these carotenoids act as pigment in xanthophores in the skin probably for mating success, in fact, Poecilia parae, a relative of the guppy fish was reported to have a clear preference on males with carotenoid colouration (48); while in females carotenoids are concentrated into eggs to potentially promote

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healthy embryonic development and promote survival of fry (9)

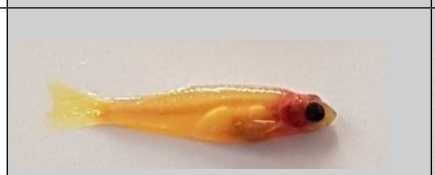
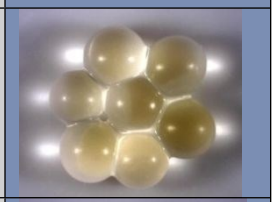
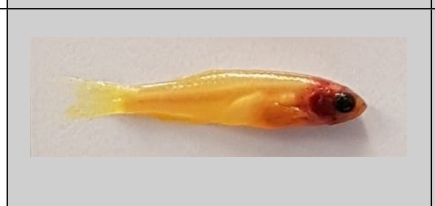
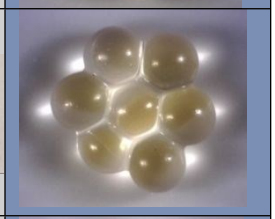
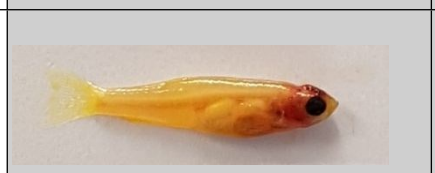
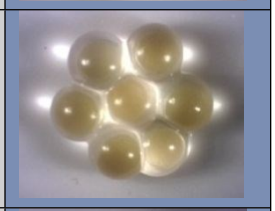
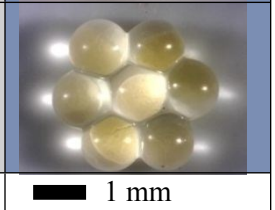
Diet	Male	Female	egg
Control			
10% SLP			
20% SLP			
50% SLP			
10% CRF			
20% CRF			
50% CRF			
Scale	1 cm		1 mm

Figure 5 Zebrafish (Casper strain) and its egg pigment variation due to carotenoid accumulation in response to carotenoid- rich diets.

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In addition to the qualitative investigations of zebrafish eggs, quantitative measurements were also carried out. As expected, eggs from fish fed a diet enriched with green-leaf derivative material had a higher concentration of carotenoids (lutein) (Table 4). HPLC analysis confirmed that out of the three carotenoids analysed (Lutein, β-carotene and astaxanthin) lutein pigments is the major carotenoid present in the eggs; the latter two carotenoids were at trace levels or undetectable.

In contrast to the results from the whole body extracts, where both CRF and SLP feeds showed significant differences ($P \leq 0.05$) compared to the control (table 4), it was only the CRF-fed fish that developed lutein-loaded eggs that were distinct ($P \leq 0.05$) from the control. It is evident that the concentration of lutein in the whole body of zebrafish is low (from around 0.1-5 mg kg $^{-1}$ D,M) compared to that of zebrafish eggs (23-66 mg kg $^{-1}$DM), with CRF-containing feeds showing greatest lutein concentration in the eggs as in Table 4. In theory, cell wall prevents partially the release of nutrients made in the pigmented (e.g. chloroplasts) tissues. CRF is a crude prepartion of chloroplasts, recoverd from spinach. Thus, chloroplastic carotenoids, lutein for example, are assumed to be better released from CRF compared to SLP.

The bright yellow/orange colour of some fish eggs, for instance, salmonids, is accounted for by the presence of carotenoids (49). These carotenoids, namely provitamin A (e.g. α and β carotene) and non-provitamin A occur in the egg yolk of zebrafish. The carotenoids are converted to retinoids (retinal) through β- carotene-15, 15-oxygenase (BCO) resulting in generation of retinoic acid necessary for various developmental processes.

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This revealed that carotenoids have essential role in the embryonic development of fish (9).

4.5. Retinol Content in Whole Zebrafish

The concentration of all-trans retinol in the experimental and control diets of zebrafish was quantified. Results indicate that all test diets had three to eight times higher ($P \leq 0.05$) retinol concentration compared with the control (Table 4). No significant difference was apparent among test diets. These results suggest that the provitamin A carotenoids from the diet were converted into retinol *in vivo* by the zebrafish.

Two types of carotene oxygenase: β -carotene-15,15-oxygenase (BCO) and β-carotene-9,10-oxygenase (BCO-II) in zebrafish was cloned by von Lintig and Vogt (50) and confirmed that the existence of these enzymes are not restricted to mammals. Von Linting and co-authors, further examined the consequence of loss of BCO function during zebrafish development and found malformations in the architecture of the branchial arch skeleton and the eye thus suggesting the significant role of BCO in zebrafish. Similar findings of BCO significance for provitamin A conversion to retinal in zebrafish was published (9).

Animals cannot synthesize retinoids *de novo* as such, they use plant derived C_{40} carotenoids as their major source for retinoids by means of enzymatic (BCO & BCO-II) cleavage (50). Dietary carotenoids absorbed through the intestinal epithelia are carried into the circulatory system in association with lipoproteins to turn them into retinoids (vitamin A). Studies suggest that retinoids are essential to fish and their deficiency causes mortality, anemia and reduced growth (51, 52). Besides the other essential roles that retinoids (vitamin A) play in vertebrates, such as vision, development, and genetic functions, they have an influence on the reproduction of zebrafish, however, storage levels of retinyl esters was apparently of little significance (52). When the importance of retinoids in the reproduction of zebrafish was examined using retinoid rich and deficient diets, reduction of whole body retinoids by 68% in females and 33% in males was obtained with the retinoid deficient diet. In addition, females fed with the retinoid deficient diets produced significantly fewer eggs than that contained retinoid or the control (52).

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5. Conclusion

Inclusion of the SLP and CRF materials into zebrafish feed formulations with reductions in fishmeal has proven successful. All experimental diets achieved equal or better dietary performance as the control, with the SLP10 and CRF10 diets achieving significantly improved FCR's. Fish showed a positive taste response to test diets. CRF is a potential new alternative feed source for partial inclusion into fish feeds, while reducing fishmeal content. CRF which can be obtained from underutilised green biomass, is abundant in nature and rich in essential nutrients; this study clearly shows that the chloroplast in the CRF material is digested by zebrafish, releasing vital nutrients that are taken up by the fish, some being converted into important components *in vivo*. Uptake of ALA and lutein, plus conversion of ALA into longer chain PUFAs was observed. Increased omega 3 FAs is a desirable outcome for commercial species, while increased lutein content may improve embryo health and survival. Using a vegetable source of PUFA (ALA) to replace fish oils seems to be of significant interest and progress in a world where livestock animals eat other animals from their own species, and are therefore more prone to disseminate their own diseases. Due to the success of this pilot study, the ready availability of material for production of CRF, and the great potential for dietary inclusion, further research is warranted. Given that CRF material is not bound by a cell well, it would be of real interest and intriguing concept to test it in a carnivorous fish feeding trial for their growth and nutritional benefits. Future work may also include assessment of increased lutein levels, due to increasing dietary inclusion of CRF, on fish fecundity, embryo fertility, hatch rate and larval survival. Since zebrafish is a good biological model for nutrient uptake, these findings may lead to the potentiality of CRF application as functional food ingredients for vulnerable populations, to fight hunger and malnutrition.

Conflicts of Interest

There are no conflicts to declare.

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