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Growth performance, fast muscle development and chemical composition of juvenile lumpfish (*Cyclopterus lumpus*) fed diets incorporating soy and pea protein concentrates

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ABSTRACT

Lumpfish (*Cyclopterus lumpus*) are widely applied as biological delousers in open net-pen farming of Atlantic salmon. As a species new to farming it is necessary to obtain a comprehensive understanding of the capacity of lumpfish to utilize plant derived feed ingredients. A feeding trial lasting for 54 days was conducted to investigate the effects of replacing fishmeal (FM) with a mix of soy protein concentrate (SPC) and pea protein concentrate (PPC) on growth, body chemical composition, and fast muscle fiber cellularity in juvenile lumpfish. Four isonitrogenous and isoenergetic diets (52 % crude protein and 14 % crude lipid) were formulated; a FM based diet was used as control (CTRL), and three experimental diets containing SPC and PPC (equal proportions of 1:1), replacing FM on weight basis at 25 % (PP25) 50 % (PP50) and 75 % (PP75). The fish grew from approximately 6.9 g to an average weight of 40.2 g in 54 days. Fish fed PP50 had significantly higher body weight, length and height compared to the other dietary groups. The whole body crude protein content of fish fed PP50 was significantly higher compared to the CTRL diet, while crude lipids were lower than those on CTRL and PP25 diets. Ash and dry matter did not differ among groups. Probability density functions showed no differences in fast muscle fiber size distributions amongst feeding groups. A higher percentage of smaller fibers in all feeding groups indicated hyperplasia was the dominant mechanism of muscle growth during the experimental period. These results suggest that a mixture of SPC and PPC can replace up to 50 % of FM in diets for juvenile lumpfish without any adverse effects on growth, chemical composition and fast muscle fiber cellularity.

1. Introduction

Two species of caligid copepods, salmon louse (*Lepoptherius salmonis,* Krøyer) and sea louse (*Caligus elongatus*) are a significant threat to farmed and wild Atlantic salmon (*Salmo salar*). Challenges associated with salmon lice have been reported since 1970 in Norway (Heuch et al., 2005), and have become the main issue for growth and expansion of Norwegian salmon production. Chemotherapeutants, such as bath treatments (hydrogen peroxide and organophosphates) or in-feed treatments (emamectin benzoate), have been used heavily to control these ectoparasites (Burridge et al., 2010). The negative impacts imposed by chemical treatments has driven the industry to use a wider selection of preventive and environmentally friendly alternatives (Powell et al., 2018). Consequently, use of alternative control strategies such as physical barriers, non-chemical baths and sea louse predators (cleaner fish) are increasing (McEwan et al., 2019).

Lumpfish, also known as lumpsucker (*Cyclopterus lumpus*), have little economic value as a food species other than use as a source of roe which is processed and sold as a substitute for caviar in fisheries across the North Atlantic regions (Davenport, 1985). Interest in the commercial production of farmed lumpfish in Norway began in 2011 (Imsland et al., 2014a), as a alternative solution to the sea-lice infestation issue. Lumpfish display cleaning symbiosis; where organisms clean cooperative host organisms, partly feeding on ectoparasites, diseased and injured tissues, and unwanted food particles (Feder, 1966). Studies performed to date have confirmed that lumpfish can be efficient delousers, reducing the mature female lice levels by 93%–97% when cocultured with farmed salmon, at a stocking density of 10%–15%

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(Imsland et al., 2014a, b; Imsland et al., 2014c). The commercial use of lumpfish for delousing has resulted in a rapid increase in their production, reaching 28.9 million fish in 2018 (Norwegian Directorate of Fisheries, 2018) making lumpfish the second largest aquaculture species in Norway. Despite increased production and usage, there is lack of published literature investigating their capacity to utilize commercially and commonly used terrestrial feed ingredients in aquafeeds.

The fish feed industry is increasing the use of plant derived ingredients (Aas et al., 2019; Ytrestøyl et al., 2015) and SPC have come to dominate feeds for Atlantic salmon, accounting for 19 % of the total feed ingredients used (Aas et al., 2019). PPC has also shown great potential as a feed ingredient for carnivorous species (Øverland et al., 2009; Zhang et al., 2012), and is currently used in limited amounts (1.3 %) in Norwegian aquafeeds (Aas et al., 2019). Incorporating plant proteins in fish feeds makes them a feasible, sustainable and cost-effective substitute to FM (Tacon and Metian, 2008). However, complete replacement of FM is still a challenge due to the imbalanced essential amino acid profile, poor palatability and presence of anti-nutritional factors (ANF's) in plant ingredients (Colburn et al., 2012; Drew et al., 2007; Urbano et al., 2000). One way to overcome the limitations of individual ingredients is to use a mixture of plant protein containing ingredients.

Even though lumpfish are not farmed for food, studies conducted on their muscle growth and development are crucial to elucidate feed effects. In most teleost fish species striated muscle predominants, are composed mainly of fast muscle fibers constituting more than 70 % of the total body mass (Sänger and Stoiber, 2001). Thus, changes in body mass are largely attributable to changes in fast muscle fiber growth, which are a consequence of variations in muscle hypertrophy (expansion in fiber diameter) and/or hyperplasia (recruitment of new muscle fibers) (Alami-Durante et al., 2010a). Muscle cellularity, the relative contributions of hypertrophy and hyperplasia to muscle growth, is affected by several factors such as egg incubation temperature (Johnston et al., 2000) and length of the photoperiod (Johnston et al., 2003). Protein is the basic component of fish feed and its level has been reported to influence fish muscle cellularity in several species (Alami-Durante et al., 2010a; Bjørnevik et al., 2003; Knutsen et al., 2019; Silva et al., 2009b). To our knowledge, the effects of plant protein ingredients on muscle development and growth of lumpfish have not been reported. Therefore, the aim of the present study was to investigate the effect of replacing FM with a mixture of SPC and PPC in feeds for juvenile lumpfish, on growth performance, fast muscle development, and whole body chemical composition.

2. Materials and methods

This feeding experiment was approved by the Ethics and Animal welfare committee at Nord University, following the Norwegian animal welfare act (LOV-2009-06-19-97).

2.1. Lumpfish and experimental set up

Juvenile lumpfish of 4 g average weight were obtained from Mørkvedbukta AS, Bodø, Norway. The fish were randomly allocated into 12 indoor rearing tanks (500 L), with 208 fish per tank, at the research station of Nord University, Bodø, Norway. Fish were acclimated to laboratory conditions for 2 weeks prior to the experiment, during which time they were fed a commercial diet (Gemma Silk, Skretting, Stavanger, Norway). Light intensity was controlled by four florescent lamps (24 h) (Grunda Viktor work lamps, 38 W, luminous flux1350 lm) facing upward. Throughout the experimental period light was dimmed to provide an illumination regime similar to that of commercial rearing practice. Fish were provided with seawater from Saltenfjorden, at 250 m depth, with a stable salinity (34‰) through a flow-through water system. Water flow rate was kept constant at 500 L/h. The temperature (7.6 ± 0.9 °C) and dissolved oxygen (86.7 ± 0.11 %) of the rearing water was monitored daily.

Table 1
Ingredient composition of the experimental diets (g 100g^{-1} diet).

Ingredients	CTRL	PP25	PP50	PP75
Fish meal [1]	58.00	43.50	29.00	14.50
Soy protein concentrate [2]	0.00	7.20	14.45	21.67
Pea protein concentrate [3]	0.00	7.20	14.45	21.67
CPSP 90 [4]	2.50	2.50	2.50	2.50
Krill meal [5]	5.00	5.00	5.00	5.00
Wheat gluten [6]	7.00	7.00	7.00	7.00
Wheat meal [7]	10.00	9.16	6.95	4.59
Pea starch [8]	5.35	5.35	5.35	5.41
Fish oil [9]	7.00	7.00	7.00	7.00
Krill oil [10]	1.50	2.25	3.05	3.85
Vitamin & Mineral Premix [11]	1.00	1.00	1.00	1.00
Lutavit E50 [12]	0.05	0.05	0.05	0.05
Antioxidant powder [13]	0.20	0.20	0.20	0.20
Sodium propionate [14]	0.10	0.10	0.10	0.10
MCP [15]	0.00	0.00	0.98	2.10
Carophyll Pink [16]	0.05	0.05	0.05	0.05
Nucleotides [17]	0.50	0.50	0.50	0.50
Garlic extract [18]	0.50	0.50	0.50	0.50
L-Histidine [19]	0.25	0.25	0.25	0.25
L-Tryptophan [20]	0.00	0.09	0.17	0.26
DL-Methionine [21]	0.00	0.00	0.35	0.70
L-Taurine [22]	1.00	1.10	1.10	1.10

CTRL: Control, PP25: 25 % of SPC and PPC inclusion, PP50: 50 % of SPC and PPC inclusion, PP75: 75 % of SPC and PPC inclusion.

[1] NORVIK LT 70 : 70.3 % crude protein (CP) 5.8 % crude fat (CF) (Sopropêche, France).

[2] Soycomil : 63 % CP, 0.8 % CF (ADM, The Netherlands).

[3] Lysamine GPS: 78 % CP, 0.9 % CF (Roquette Frères, France).

[4] Soluble fish protein hydrolysate: 82.6 % CP, 9.6 % CF (Sopropêche, France).

[5] 61.1% CP, 17.4 % CF (Aker Biomarine, Norway).

[6] VITAL: 83.7 % CP, 1.6 % CF, (Roquette, Frères, France).

[7] 10.2% CP; 1.2 % CF (Casa Lanchinha, Portugal).

[8] NASTAR 90 % starch, (Cosucra, Belgium).

[9] (SAVINOR UTS, Portugal).

[10] (Aker Biomarine, Norway).

[11] Vitamins (IU or mg kg-^{1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg-1 diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings (PREMIX Lda, Portugal).

[12] (ROVIMIX E50, DSM Nutritional Products, Switzerland).

[13] Paramega PX (Kemin Europe NV, Belgium).

[14] Disproquímica (Portugal).

[15] ALIPHOS MONOCAL, 22.7 % P (ALIPHOS, Belgium).

[16] Carophyll Pink 10 % CWS (DSM Nutritional Products, Switzerland).

[17] Nucleoforce Salmonids (BioIbérica, Spain).

[18] Macrogard, 67.2 % beta-glucans (Biorigin, Brazil).

[19] L-Histidine 98 %, (Ajinomoto Eurolysine SAS, France).

[20] L-Tryptophan 98 %, (Ajinomoto Eurolysine SAS, France).

[21] DL-METHIONINE FOR AQUACULTURE 99 %, (EVONIK Nutrition & Care GmbH, Germany). [22] L-Taurine 98 %, (ORFFA, The Netherlands).

2.2. Experimental diets and growth trial

The feed ingredient composition, calculated and analyzed proximate composition of the experimental diets are presented in Tables 1 and 2, which were manufactured by SPAROS Lda. Olhao, Portugal. The diets were formulated to be isoproteic and isoenergetic on the basis of crude protein and gross energy content. A FM based diet was used as control (CTRL) and three experimental diets were formulated to replace

Table 2
Calculated and analyzed proximate nutrient composition of the experimental diets on a as fed basis (%).

	CTRL	PP25	PP50	PP75
Calculated				
Crude protein	53.9	53.9	53.9	53.9
Crude fat	13.4	13.4	13.4	13.4
Fiber	0.3	0.7	1.0	1.3
Starch	9.2	9.5	8.8	8.1
Ash	11.3	9.5	8.2	7.1
Gross Energy	20.0	20.2	20.3	20.4
Arginine	3.5	3.7	4.0	4.2
Histidine	1.4	1.4	1.4	1.4
Isoleucine	2.0	2.1	2.2	2.4
Leucine	3.8	3.9	4.0	4.1
Lysine	3.9	3.9	3.9	3.9
Tryptophan	0.5	0.5	0.5	0.5
Threonine	2.5	2.3	2.2	2.1
Valine	2.5	2.5	2.6	2.6
Methionine + Cysteine	2.3	2.0	2.0	2.0
Phenylalanine + Tyrosine	4.5	4.5	4.5	4.5
Taurine	1.2	1.2	1.2	1.2
Total Phosphorous	1.7	1.5	1.4	1.4
Vitamin C (mg/kg)	1000.0	1000.0	1000.0	1000.0
Vitamin E (mg/kg)	350.0	350.0	350.0	350.0
Eicosapentaenoic acid (EPA)	1.6	1.6	1.6	1.6
Docosahexaenoic acid (DHA)	2.0	1.9	1.8	1.6
EPA + DHA	3.5	3.4	3.4	3.4
Total phospholipids	2.6	2.6	2.6	2.6
Analyzed				
Dry matter	93.9	94.9	95.3	93.3
Crude protein	51.1	52.1	52.5	52.4
Crude fat	14.9	14.8	14.4	14.5
Ash	11.3	9.7	8.5	6.2
Gross Energy	20.5	20.8	20.8	20.8

25 % (PP25) 50 % (PP50) and 75 % (PP75) of the FM with a mixture of SPC and PPC (1:1 proportion). The remaining protein ingredients such as wheat gluten, krill meal, and CPSP 90 were kept constant. The diets were supplemented with L-tryptophan, DL-methionine, L-taurine and L-histidine to keep these ingredients similar among all diets. Wheat meal was used to balance the starch and carbohydrate content among the diets. Krill oil was used in increasing levels from CTRL to the PP75, to increase the content of EPA, DHA and phospholipids.

All dry ingredients were mixed in a double-helix mixer (model RM90, MAINCA Spain) passed through a 0.4 mm micro-pulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets were extruded in a twin-screw extruder (model BC45, Clextral, France) with a 1.5 mm die and extruded pellets dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). Oils were added post-extrusion by vacuum coating (model PG-10VCLAB, Dinnissen, Netherlands). Experimental diets were stored at room temperature until they were used for feeding. The four diets were randomly allocated to triplicate tanks (n = 3 / feed group), and each tank was equipped with an automatic feeder (ArvoTec, Sterner, Norway). Fish were fed the experimental diets to apparent satiation, with the feeding rate of 2.5 % of their body mass. The feeding were closely monitored through visual inspections and the feed were provided eight time points every day, between 6:00 to 21:00 during the 54 day experimental period.

2.3. Sample collection

At the beginning and end of the growth trial, all fish were individually weighed to the nearest 0.5 g and their standard length and body height measured to the nearest 0.1 mm. In addition, at each sampling point liver and visceral weight were also recorded. A total of 20 fish per tank were sampled randomly for chemical composition analysis. Fish were pooled into 10 fish per pool and 2 pooled samples per tank (n = 6 fish / feed group), packed in plastic bags, and frozen at -40 °C until further analysis. Five fish were sampled per tank and used for the evaluation of muscle histology. All samples were taken at the start, 19, 35, and 54 days (19D, 35D, 54D) of the growth trial. Prior to sampling, fish were anaesthetized with MS-222 (Tricaine methane sulphonate; Argent Chemical Laboratories, USA; 30 g /L) and dispatched by a sharp blow to the head.

2.4. Biochemical analyses

The frozen whole fish samples were thawed for approximately 5 h at 4 °C and pooled samples were homogenized using a conventional food processor (Bosch GmbH, CNCM11, Slovenia). Part of this homogenate was used to determine the dry matter and ash content in whole fish. Remaining homogenate was freeze dried for 96 h at −70 °C (VirTis benchtop K Mod, Warminster, U.S.A) and dry matter was recorded. The freeze dried samples were frozen at −80 °C before being re-ground (3 × 15 s) into a fine powder for crude protein and crude fat (dry basis) analysis. The proximate composition of the feed pellets was also determined. In brief, moisture content was determined by drying whole fish (2.0 g) and feed (5.0 g) samples to a constant weight at 104 °C for 20 h (ISO 6496-1999). The whole fish samples were combusted in a muffle furnace to a constant weight at 540 °C for 16 h to determine the ash content at FBA, whereas, the feed was analysed by Eurofins (Moss, Norway) (ISO 5984-2002). Crude protein of fish and feed were determined from a 0.5 g samples using the Kjeldahl titration method (N x 6.25, KjeltecTM 2300, Foss Tecator AB, Höganäs, Sweeden ISO 5983-1987). Crude fat was determined gravimetrically using 2.0 g of freeze dried fish and 5.0 g of feed samples using the diethyl ester extraction method, according to the (Norwegian Standard Association., 1994) and feed energy analysed by bomb calorimeter (IKA C200, Staufen, Germany: ISO 9831: 1998). All biochemical analyses of the feed and whole fish were triplicated and duplicated respectively.

2.5. Fast muscle cellularity

To evaluate the muscle cellularity, a 5 mm thick cross sectional steak was cut just anterior to the second dorsal fin of juvenile lumpfish (Fig. 1) and photographed together with graph paper to measure the total fast muscle cross-sectional area (TCA) of the steak (SigmaScan pro. 5.0, Systat, Inc.). Depending on fish size, two to three muscle blocks (5 × 5 × 5 mm) from the dorsal left side of each fillet were taken for histological analysis. In brief, muscle blocks were mounted on cork sheets (1.5 × 1.5 cm) covered in cryomatrix (Shandon Cryomatrix, Thermo scientific) and frozen in 2-methyl butane (60 s) cooled to near its freezing point (−159 °C) in liquid nitrogen. Frozen blocks were stored at −80 °C until further analysis. Muscle blocks were sectioned (7 μm) at −18 °C in a cryostat (Cryostar NX50, Thermo Scientific, USA), air dried and stained with hematoxylin (Harris hematoxylin, Sigma Aldrich, Steinheim, Germany). The outlines of the muscle fibers (area) of 800 fibers per fish were examined using a light microscope (Axioscop 2 mot plus; Carl Zeiss INC., Germany) equipped with a camera, and area measured using the software Axio Vision (Rel.4.2, Carl Zeiss INC., Germany). All the parameters measured for muscle cellularity were normalized based on the size of fish, as described by Alami-Durante et al. (2010a).

2.6. Calculations

Condition factor (B^1) was calculated according to the formula proposed by Richter et al. (2000). B^1 (g cm^{-3}) = fish weight (g) / [fork length (cm) x body height ^{2} (cm)]. Somatic indices and Specific Growth Rate (SGR) were calculated employing the following formulae: Hepatosomatic index (HSI) = [liver weight (g) / fish weight (g)] × 100. Visero-somatic index (VSI) = [visceral weight (g) / fish weight (g)] × 100. SGR (% day^{-1}) = 100 × ln [final mean weight (g) - initial mean weight (g)] / number of feeding days.

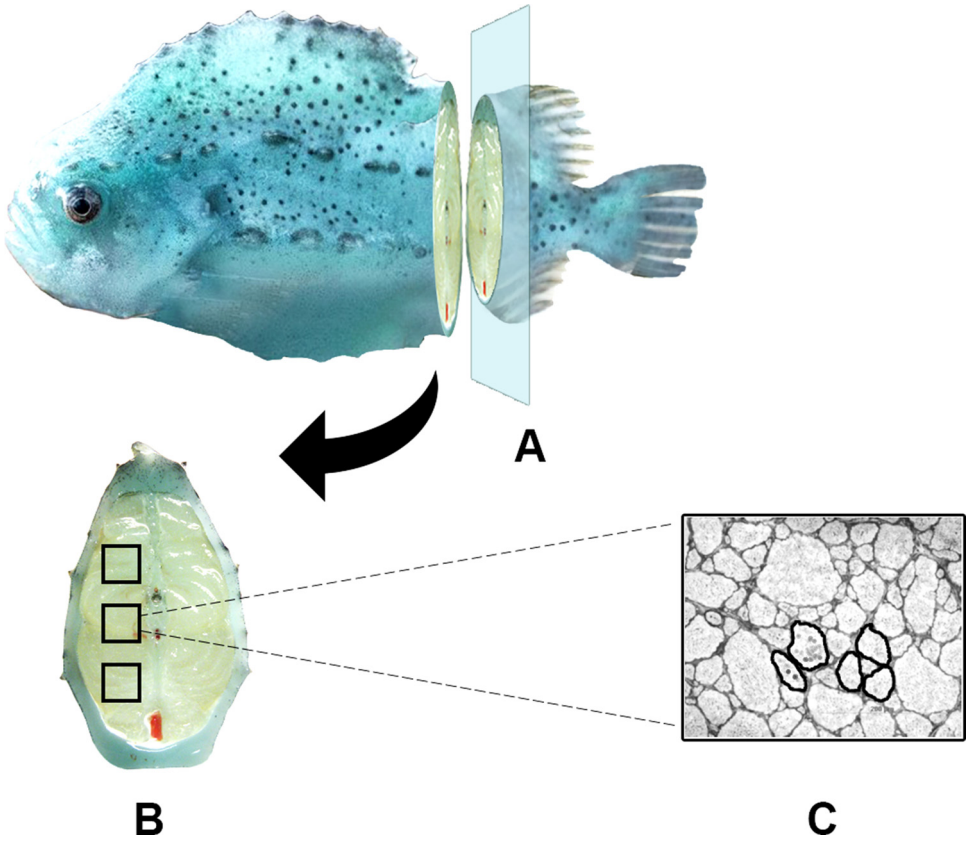

Fig. 1. A) Schematic view of sampling locations in lumpfish, B) sample sites of muscle blocks from the dorsal left side of the steak, C) fast muscle histological section (×10) highlight of the individual muscle fiber area measured.

2.7. Statistical analysis

The software Sigmaplot 14.0 (Systat software, San Jose, CA) was used for the statistical analyses. A Shapiro-Wilk test was used to assess the normality of distributions, and the Brown-Forsythe F-test to determine the equality of group variances. One way analysis of variance (ANOVA) was performed for the parametric data. Significant differences revealed in ANOVA were followed by Tukey's multiple comparison test. A Kruskal-Wallis one-way analysis of variance on ranks, followed by Tukey's multiple comparison test was used for the nonparametric data. Dunn's pairwise multiple comparison test was used only to assess the significance of the unequal size of growth-related data at the end of the experiment. Distribution of muscle fiber diameter was evaluated using smooth non parametric distributions where 800 measurements of fast fiber diameters were fitted using a kernel function (Bowman and Azzalini, 1997; Johnston et al., 1999). Experimental groups compared at the end of feeding period were of similar body length (n = 12 per group). Comparison of the distribution of muscle fiber diameters was done by applying the nonparametric Kolmogorov-Smirnov test, with the null hypothesis that one-dimensional probability density functions (PDF) of groups were equal over all the diameters. Bootstrap techniques were used to create the variability bands around the group PDFs using the mean smoothing parameter. This was used to identify which areas of the muscle fiber distribution of diameters contributed to significant differences. Significance was established when $p < 0.05$; data is presented as means ± SEM.

3. Results

3.1. Chemical composition of the experimental diets

Minor differences were observed between the calculated and analyzed proximate composition of the diets (Table 2). The chemical analysis showed that crude protein was slightly lower and crude lipid was slightly higher than the calculated values, while ash and energy were similar to the calculated values.

3.2. Growth performance

The experimental diets were well accepted and no mortalities were recorded. The final weight of fish increased 5–6 fold their initial weight (Table 3). Fish fed PP50 had significantly higher body weight, length and height compared to the other diets at the end of the experiment. The height of the fish increased from an average of 2.22 cm–4.22 cm during the course of the experiment. Length of the fish appeared to be proportional with weight gain and was significantly higher for fish fed PP50 compared to all other diet groups. The fish fed PP50 diet tended to have higher SGR ($p = 0.06$) compared to the other feding groups at the end of the feeding period. The B^1 ranged between 0.23 to 0.33 and was slightly, but significantly higher in fish fed plant diets than those on the CTRL diet at the end of the experiment. No significant differences were found for the VSI among the feeding groups. HSI varied from 2.1–2.5 with the lowest value for fish receiving PP25 and highest for fish on the PP75 diet ($p < 0.05$).

3.3. Chemical composition of fish

Crude fat, protein, ash and dry matter of whole body increased slightly during the experimental period (Table 4). The crude fat content of all groups showed a small numerical and temporal drop after being introduced to the experimental diets compared to the initial levels ($p > 0.05$). However, crude fat content increased for all four groups after 19 days on experimental diets. The CTRL group showed a higher crude fat content at the end of the experiment compared to PP25 and

Table 3
Growth parameters and condition indices of lumpfish fed diets with different levels of plant protein concentrates.

Parameter	Feeding trial period	Plant protein inclusion levels				p - Value
		CTRL	PP25	PP50	PP75	
Growth parameters						
Body weight (g)	Start (0 days)	6.88 ± 0.06	6.80 ± 0.06	6.83 ± 0.06	7.03 ± 0.06	0.246
	Continuous phase I (19D)	14.63 ± 0.31^{ab}	14.25 ± 0.24^{ab}	15.09 ± 0.26^{a}	13.72 ± 0.34^{b}	0.021
	Continuous phase II (35D)	26.34 ± 0.57	25.67 ± 0.59	26.92 ± 0.51	26.5 1 ± 0.63	0.286
	End (54 D)	40.75 ± 0.56^{b}	40.58 ± 0.59^{b}	46.26 ± 0.68^{a}	35.84 ± 0.94^{c}	< 0.001
Body length (cm)	Start (0 days)	4.59 ± 0.01	4.61 ± 0.02	4.64 ± 0.01	4.71 ± 0.07	0.432
	Continuous phase I (19D)	6.24 ± 0.05^{a}	6.20 ± 0.03^{a}	6.22 ± 0.04^{a}	6.03 ± 0.05^{b}	0.005
	Continuous phase II (35D)	7.65 ± 0.06^{ab}	7.59 ± 0.06^{ab}	7.72 ± 0.05^{a}	7.51 ± 0.06^{b}	0.043
	End (54 D)	8.69 ± 0.04^{b}	8.55 ± 0.05^{b}	8.91 ± 0.05^{a}	7.91 ± 0.08^{c}	< 0.001
Body height (cm)	Start (0 days)	2.23 ± 0.01	2.23 ± 0.01	2.22 ± 0.01	2.26 ± 0.01	0.283
	Continuous phase I (19D)	3.06 ± 0.03	3.07 ± 0.02	3.09 ± 0.02	3.05 ± 0.03	0.726
	Continuous phase II (35D)	3.78 ± 0.03^{c}	3.76 ± 0.03^{b}	3.82 ± 0.03^{abc}	3.91 ± 0.04^{a}	0.008
	End (54 D)	4.07 ± 0.03^{b}	4.27 ± 0.03^{bc}	4.48 ± 0.03^{a}	4.07 ± 0.05^{b}	< 0.001
SGR (% day $^{-1}$)	Start (0 days)	n.a	n.a	n.a	n.a	
	Continuous phase I (19D)	3.94 ± 0.14	3.89 ± 0.17	4.18 ± 0.002	3.49 ± 0.21	0.074
	Continuous phase II (35D)	3.84 ± 0.13	3.78 ± 0.07	3.92 ± 0.06	3.72 ± 0.11	0.579
	End (54 D)	3.30 ± 0.03	3.32 ± 0.05	3.55 ± 0.05	3.13 ± 0.16	0.062
Condition indices						
HSI	Start (0 days)	2.53 ± 0.08	2.58 ± 0.09	2.59 ± 0.08	2.44 ± 0.10	0.395
	Continuous phase I (19D)	2.47 ± 0.05	2.50 ± 0.11	2.27 ± 0.05	2.40 ± 0.07	0.066
	Continuous phase II (35D)	2.22 ± 0.05	2.20 ± 0.04	2.14 ± 0.04	2.26 ± 0.05	0.259
	End (54 D)	2.37 ± 0.06^{ab}	2.18 ± 0.04^{b}	2.22 ± 0.04^{b}	2.48 ± 0.04^{a}	< 0.001
VSI	Start (0 days)	12.84 ± 0.19	12.57 ± 0.20	12.83 ± 0.19	12.49 ± 0.31	0.210
	Continuous phase I (19D)	15.18 ± 0.22	15.32 ± 0.22	14.94 ± 0.33	14.56 ± 0.31	0.202
	Continuous phase II (35D)	13.86 ± 0.19	13.30 ± 0.26	13.04 ± 0.25	13.54 ± 0.21	0.086
	End (54 D)	13.72 ± 0.28	13.34 ± 0.17	13.51 ± 0.33	13.23 ± 0.19	0.144
Condition factor B[1] (g cm^{-3})	Start (0 days)	0.30 ± 0.001	0.30 ± 0.001	0.30 ± 0.001	0.30 ± 0.001	0.417
	Continuous phase I (19D)	0.25 ± 0.002^{b}	0.24 ± 0.002^{a}	0.25 ± 0.002^{b}	0.24 ± 0.002^{a}	0.001
	Continuous phase II (35D)	0.24 ± 0.001^{a}	0.24 ± 0.001^{a}	0.24 ± 0.00 ^{a}	0.23 ± 0.001^{b}	0.001
	End (54 D)	0.28 ± 0.002^{a}	0.32 ± 0.060^{b}	0.30 ± 0.050^{b}	0.33 ± 0.020^{b}	0.001

CTRL: Control, PP25: 25 % of SPC and PPC inclusion, PP50: 50 % of SPC and PPC inclusion, PP75: 75 % of SPC and PPC inclusion. Values represented as means ± SEM. Growth parameters and CF for week 0 are based on measurements of all fish. Similarly, growth parameters and CF at the end of the feeding trial based on both fish sampled and fish remaining after 54 days. Significant differences between treatment groups at the same time point indicated with different superscript letters ($p < 0.05$).

PP50 ($p < 0.05$). Additionally, the whole body crude protein content was higher in the PP50 group compared to the CTRL at the end of the experiment ($p < 0.05$).

3.4. Fast muscle cellularity

No differences were found for muscle cellularity among the diet groups, except for mean diameter and muscle fiber size category ranging from 50 µm to 70 µm (Table 5). At the start of the experiment, fish had an average fast muscle fiber number of 62659 ± 4645 and increased for all groups close to five fold during the experimental period. The daily recruited muscle fiber numbers were numerically higher in the PP50 group (4768) compared to the control (4274), PP25 (3882) and PP75 (4283). Size distribution of fast muscle fibers were categorized into 7 groups. Juvenile lumpfish showed a higher number of smaller fibers ranging from 10 to 70 µm and fewer of the larger fibers in the size range 90 − 120 µm. The fibers with diameters $10 < D \leq 30$ µm were the most common and abundant in all diet groups. The only significant difference among diets were for fiber diameters ranging from $50 < D \leq 70$ µm that were lower in fish fed PP75 compared to the other groups. The PDFs for fiber diameter distribution showed, however, no differences among the feeds (Fig. 2, $p > 0.05$).

4. Discussion

In the present study, the utilization of the plant protein concentrates of SPC and PPC in diets for juvenile lumpfish were evaluated based on growth performance, body chemical composition and muscle development.

4.1. Fish growth performance

In the wild, lumpfish feed on a variety of prey items including, plankton, jellyfish and polycheates (Daborn and Gregory, 1983; Davenport, 1985; Ingólfsson and Kristjánsson, 2002; Mitamura et al., 2012), as well as seaweeds and seagrass (Davenport, 1985). Lumpfish have the ability to switch their natural prey choice to whatever is available (Imsland et al., 2015a, b). This opportunistic feeding behavior combined with a gut length twice the body length (Davenport, 1985) indicate that lumpfish are omnivorous and may explain why the best growth performance (i.e. body weight, length, and height) was observed in fish fed the PP50 diet. The experiment was not designed to study feed intake, but all the groups were fed in excess to secure *ad libitum* feed intake, assumed to promote fast growth and maximize utilization of the feed. With regard to delousing, smaller juvenile stages (initial weight of 20 g) are more efficient compared to larger conspecifics (Imsland et al., 2016). Therefore, in order to achieve optimal

Table 4
Chemical composition [%] of whole body of lumpfish fed diets with different inclusion levels of plant protein concentrates.

Parameter	Feeding trial period	Plant protein inclusion levels				p - value
		CTRL	PP25	PP50	PP75	
Dry matter (%)	Start (0 days)	13.33 ± 0.13	13.05 ± 0.10	13.19 ± 0.17	12.89 ± 0.15	0.190
	Continuous phase I (19D)	13.40 ± 0.08	13.29 ± 0.09	13.32 ± 0.05	12.90 ± 0.18	0.138
	Continuous phase II (35D)	13.75 ± 0.12	13.37 ± 0.11	13.24 ± 0.14	13.29 ± 0.16	0.056
	End (54 D)	14.30 ± 0.12	13.79 ± 0.15	14.03 ± 0.08	13.94 ± 0.14	0.057
In dry matter, %						
Crude protein	Start (0 days)	60.47 ± 0.38	60.79 ± 0.34	59.76 ± 0.21	60.50 ± 0.33	0.255
	Continuous phase I (19D)	60.47 ± 0.38	60.89 ± 0.46	61.28 ± 0.12	61.54 ± 0.79	0.474
	Continuous phase II (35D)	61.16 ± 0.46	61.46 ± 0.67	61.75 ± 0.33	62.68 ± 0.77	0.373
	End (54 D)	60.67 ± 0.22^{b}	61.06 ± 0.39^{ab}	62.20 ± 0.24^{a}	61.16 ± 0.17^{ab}	0.012
Crude lipid	Start (0 days)	18.58 ± 0.29	18.17 ± 0.39	18.71 ± 0.46	18.27 ± 0.37	0.731
	Continuous phase I (19D)	17.59 ± 0.05	16.32 ± 0.52	17.29 ± 0.14	16.03 ± 0.66	0.108
	Continuous phase II (35D)	19.26 ± 0.77	18.04 ± 0.22	18.40 ± 0.37	17.14 ± 0.84	0.278
	End (54 D)	21.08 ± 0.44^{a}	19.27 ± 0.46^{b}	18.98 ± 0.38^{b}	20.13 ± 0.30^{ab}	0.006
Ash	Start (0 days)	1.41 ± 0.09	1.31 ± 0.04	1.45 ± 0.12	1.37 ± 0.08	0.670
	Continuous phase I (19D)	1.44 ± 0.14	1.47 ± 0.12	1.44 ± 0.12	1.42 ± 0.15	0.904
	Continuous phase II (35D)	1.62 ± 0.04	1.60 ± 0.01	1.58 ± 0.02	1.62 ± 0.03	0.624
	End (54 D)	1.62 ± 0.02	1.63 ± 0.03	1.66 ± 0.02	1.62 ± 0.02	0.716

CTRL: Control, PP25: 25 % of SPC and PPC inclusion, PP50: 50 % of SPC and PPC inclusion, PP75: 75 % of SPC and PPC inclusion. Values represented as means ± SEM (n = 6 / treatment). Significant differences between treatment groups at the same time point indicated with different superscript letters ($p < 0.05$).

delousing, it is essential to maintain their uniform and slow to moderate growth during the process of co-culture with hosts (Imsland et al., 2018). Fast growth rate is not desirable in lumpfish in the pens with the salmon, because fish larger than 350 g become less interested in louse (Imsland et al., 2014b). Growth performance is, however, established as a parameter to evaluate the efficiency of alternative feed ingredients (Shearer, 2000) and is considered to be an important welfare indicator (Huntingford and Kadri, 2014).

The experimental diets were optimized to be isoenergetic and isoproteic. Assuming that fish were fed to satiation, reduced weight gain for fish fed the PP75 diet may be explained by the reduced utilization of energy or nutrients provided in this diet. Reduced utilization of plant based diets may be explained by ANF's palatability, as well as modified energy metabolism. ANF's such as lectins, saponins, glucosinolate and oligosaccharide are removed from the SPC (Colburn et al., 2012; Drew et al., 2007), while phytate and non-starch polysaccharides (NSP's) are still present in SPC as well as in PPC (Collins et al., 2013; Storebakken et al., 1998). Phytate is known to interfere with mineral absorption and growth (Baeverfjord et al., 2019). Atlantic salmon fed 50 % of untreated SPC diet showed reduced whole body element concentrations (Ca, Mg, and Zn) and a lower apparent digestibility coefficient of the same elements compared to the phytase treated SPC diet (Storebakken et al., 1998). Air classified PPC also contain saponins (Penn et al., 2011), and may result in growth arrest (González-Rodríguez et al., 2016; Tian et al., 2018), associated with histopathology of the distal intestine (Krogdahl et al., 2015). A study with Atlantic salmon fed 35 % air classified PPC in their feed resulted in reduced weight gain, with SGR and enteropathy present in the distal intestine (Penn et al., 2011). Research with the omnivore sharp-snout sea bream (*Diplodus puntazzo*) showed that inclusion of PPC at 160 and 320 g kg^{-1} gave poor growth, and alterations in the distal intestinal morphology associated with ANFs in the PPC (Nogales-Mérida et al., 2016). Hence, plant protein ingredients may be less palatable (Grey et al., 2009), and have a negative effect on feed intake (Kader and Koshio, 2012; Takakuwa et al., 2019). The diets in the present experiment were supplemented with feed attractants in order to enhance their acceptability and growth performance. Nucleotides (Burrells et al., 2001), krill meal (Hatlen et al., 2017; Kousoulaki et al., 2013; Zhang et al., 2012) and soluble fish

Table 5
Fast muscle cellularity of lumpfish; data normalized by total length.

	Start	End (54 D)				p - value
		CTRL	PP25	PP50	PP75	
Fiber number	62659 ± 4645	310233 ± 20243	284228 ± 21793	331334 ± 26872	298406 ± 20353	0.528
D mean	35.2 3 ± 1.18	33.47 ± 0.55^{a}	32.77 ± 0.83^{ab}	32.13 ± 1.11^{ab}	31.48 ± 0.73^{b}	0.047
D median	30.71 ± 1.63	25.97 ± 0.62	25.16 ± 0.79	24.59 ± 0.77	24.89 ± 0.75	0.376
D max	117.66 ± 14.64	171.38 ± 17.07	175.33 ± 21.23	163.72 ± 18.15	142.16 ± 4.98	0.113
D mean of upper 95th percentile	75.79 ± 2.31	81.77 ± 1.95	80.19 ± 2.73	80.12 ± 3.52	78.24 ± 1.18	0.185
Proportion (%) white muscle fibers with						
D ≤ 10 µm	3.80 ± 3.05	6.60 ± 0.97	5.35 ± 0.72	7.13 ± 1.25	6.33 ± 1.14	0.743
10 < D ≤ 30 µm	45.15 ± 7.47	52.54 ± 1.64	54.84 ± 1.57	55.34 ± 1.15	55.97 ± 1.97	0.210
30 < D ≤ 50 µm	30.21 ± 5.88	21.85 ± 1.60	22.67 ± 1.51	21.05 ± 1.36	22.21 ± 1.68	0.854
50 < D ≤ 70 µm	13.98 ± 3.77	11.08 ± 0.79^{a}	9.57 ± 0.71^{ab}	9.06 ± 0.81^{ab}	8.21 ± 0.71^{b}	0.046
70 < D ≤ 90 µm	5.37 ± 1.99	4.13 ± 0.40	4.19 ± 0.36	3.78 ± 0.42	4.03 ± 0.29	0.639
90 < D ≤ 120 µm	1.18 ± 0.99	2.68 ± 0.22	2.37 ± 0.26	2.46 ± 0.26	2.52 ± 0.13	0.607
D > 120 µm	0.29 ± 0.95	1.10 ± 0.22	0.99 ± 0.27	1.72 ± 0.35	0.71 ± 0.13	0.115

CTRL: Control, PP25: 25 % of SPC and PPC inclusion, PP50: 50 % of SPC and PPC inclusion, PP75: 75 % of SPC and PPC inclusion.
Values are represented as mean ± SEM. Raw means for the end of the feeding period with different superscript letters differ significantly ($p < 0.05$).

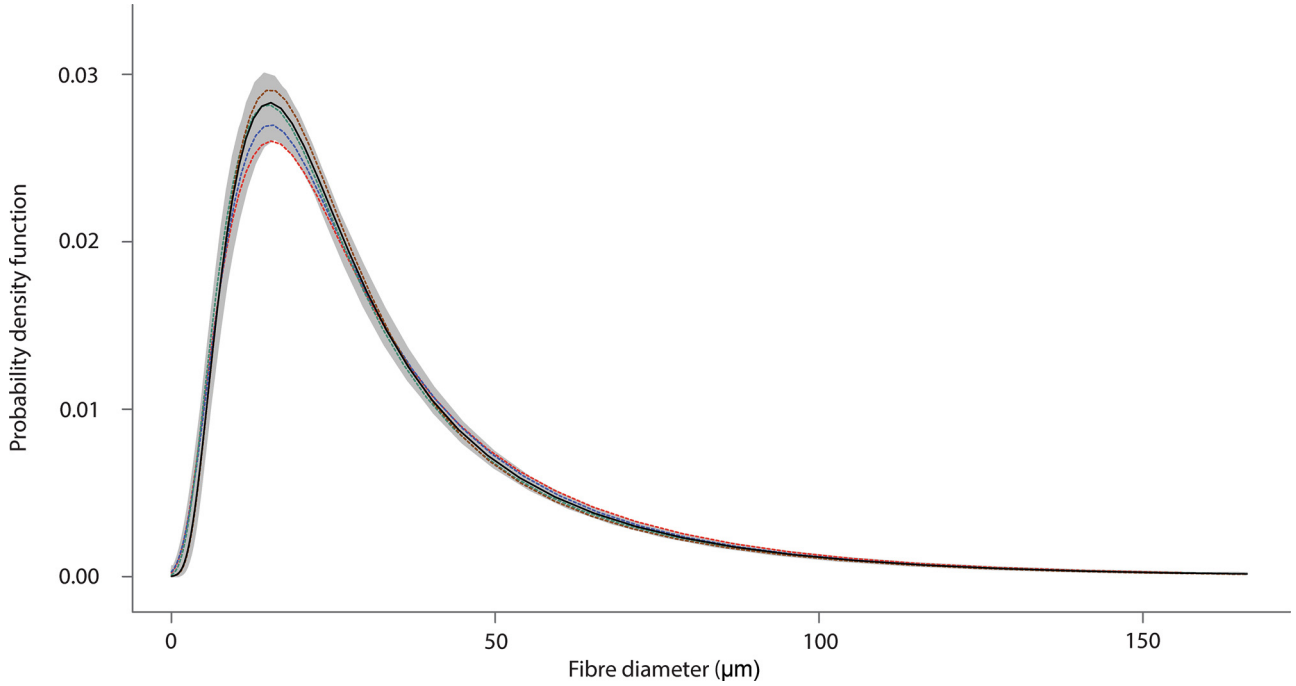

Fig. 2. Bootstrapping analysis comparing all four groups over all fast fiber diameters. Probability density functions of mean fiber distribution in juvenile *C. lumpus* fed mix of SPC and PPC diets showing in; red (CTRL), blue (PP25), green (PP50) and brown (PP75) represent in dotted lines. Black solid line represent the overall mean of all four groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

protein concentrates (Kousoulaki et al., 2009, 2012) in the diets of Atlantic salmon and rainbow trout are known to have growth promoting effects.

Length-weight relation is used to monitor growth and to evaluate the nutritional status or condition of the fish (Jones et al., 1999). In the present study, length and height were doubled with the 5–6 fold increase in weight. The three dimensional growth pattern suggests that condition factor (CF) should not only be based on weight and length, but also height. The CF commonly reported in the scientific literature is mainly calculated based on Fulton's condition factor (1911), $K = 100\,wt\,(g) \times length\,(cm)^{-3}$. The K values at termination of the experiment were 6.15 ± 0.05, 6.37 ± 0.05, 6.44 ± 0.05 and 6.79 ± 0.06 for the CTRL, PP25, PP50 and PP75, respectively. These K-values were higher than the values of 2.6–4.2 reported earlier for lumpfish (50 % crude protein) (Imsland et al., 2018), suggesting that the fish were in a good nutritional condition. All K values were slightly reduced at termination compared to the start of the experiment, with the highest value noted for the PP75 diet and lowest for the CTRL. This is simillar to results reported by Imsland et al. (2018) were the highest K also was found for the group with lower weight gainIn conjugation with K values, the B^1 showed lower values for the CTRL diet compared to the other three experimental diets. Furthermore, B^1 did not show any decline in value between the start and end of the experiment, suggesting that B^1 may be a more robust measure than the traditional K value and should be considered in future studies with lumpfish. Fish liver is the major organ with respect to nutrient metabolism, producing bile-salts and storing lipid and glycogen (Brusle and Anadon, 1996). Liver size varies a lot among fish species and HSI can range from 1.2 to 1.6 in Atlantic salmon (Gong et al., 2019; Kiron et al., 2016; Sørensen et al., 2017) and up to 9–11 in Atlantic cod (Ingebrigtsen et al., 2014) depending on energy intake (Hatlen et al., 2007). The large liver in cod reflects its importance in storage of lipid; up to 80 % of the lipid content can be found in the liver (Albrektsen et al., 2006). The HSI values in lumpfish in the present experiment were higher than usually found in Atlantic salmon, but still in the lower range of Atlantic cod. The higher HSI in fish fed PP75 is in line with a study performed with juvenile gilthead sea bream, where HSI was higher (0.87 versus 0.80; $p < 0.05$) in fish on a diet where 75 % of FM was replaced with a mixture of corn gluten meal, wheat gluten, extruded peas, rapeseed meal and extruded whole wheat compared with those on FM diet (De Francesco et al., 2007). In contrast, studies with seabass (*Dicentrarchus labrax*) showed no effect on HSI when more than 50 % of FM was replaced with plant protein mixtures in their diets (Kaushik et al., 2004).

4.2. Chemical composition

The higher content of whole body protein in fish fed PP50 compared to those fed the CTRL diet confirm the higher growth of this group, as body protein is a key predictor for gain of body weight (Dumas et al., 2007). A correlation between protein deposition and body weight has also been reported for rainbow trout (Brinker and Reiter, 2011; Dumas et al., 2007). Crude lipid content of whole body decreased below the initial levels after introduction of the experimental diets. This observation indicates that lumpfish were in a negative energy balance and used body lipid during the acclimation period before they fully accepted the experimental diets. Fat seems to be the preferred energy source over protein in anorectic lumpfish.

The whole body lipid content showed minimal increase during the course of the experiment for all diet groups, indicating that lumpfish are not depositing much lipid in body tissues and organs. The low body lipid content should be reflected in the diets of this species. The significantly higher lipid content in lumpfish fed the CTRL compared to groups fed PP25 and PP50 indicated that lipid or energy utilization was affected by incorporation of plant protein concentrates in the diets. Altered lipid metabolism in fish fed plant proteins have been reported in a number of other studies such as Atlantic salmon fed air classified faba bean protein concentrate at 50 to 200 g kg^{-1} (De Santis et al., 2015), gilthead sea bream fed 100 % of rapeseed protein concentrate and 100 % SPC (Kissil et al., 2000), yellow croaker (*Larimichthys crocea*) fed 100 % SPC (Wang et al., 2017), and Senegalese sole (*Solea senegalensis*) fed plant protein mix ranged from 70 to 80% (Silva et al., 2009a). The changes in lipid metabolism may be explained by reduced re-absorption of bile acids (Romarheim et al., 2006; Sørensen et al., 2011) resulting in lower lipid digestibility, and reduced cholesterol in fish

plasma (Dias et al., 2005; Kortner et al., 2013). However, further studies are needed to establish the requirement for lipid in diets, and the relationship between lipid metabolism and plant protein ingredients in juvenile lumpfish.

4.3. Muscle cellularity

The muscle fiber distribution, analysed using both the PDFs and the muscle fiber size classes, illustrates that the growth of juvenile lumpfish mainly takes place through hyperplastic growth. The fast muscle fiber data in all groups showed a similar fiber distribution, being dominated by fast fibers of $< 30\,\mu m$, following a sharp decline in the presence of muscle fibers $> 30\,\mu m$. This is not unique to lumpfish but is ubiquitous, being described in several other juvenile fish species such as Atlantic salmon (Bjørnevik et al., 2003; Higgins and Thorpe, 1990), white seabass (*Atractoscion nobilis*) (Zimmerman and Lowery, 1999), Senegalese sole (Valente et al., 2016), and rainbow trout (Alami-Durante et al., 2010a). The significant differences observed for muscle fibers with diameters between $50 < D \leq 70\,\mu m$ of fish fed PP75 vs. CTRL diet were not supported by the PDFs. Fiber population analysis is a stronger statistical tool than individual measurements (Johnston et al., 1999), suggesting that diet had no effect on the fiber size distribution or fiber number. Similar results have been reported from feed experiments on Atlantic salmon with no or only minor influence on muscle cellularity (Bjørnevik et al., 2003; Johnston et al., 2002). In contrast, fiber analysis showed that the size distribution of fast muscle fibers of juvenile blackspot seabream (*Pagellus bogaraveo*) fed a protein rich diet favored muscle growth by hyperplasia (Silva et al., 2009b).

Relatively few studies have attempted to elucidate the effect of plant protein sources or FM replacement by plant ingredients on muscle cellularity (Alami-Durante et al., 2010a, b; Knutsen et al., 2019). Sensitivity to dietary protein source and amino acid profile was found in juvenile rainbow trout on a diet of high soybean meal inclusion. This resulted in a lower median fiber diameter of white muscle than fish with high wheat and pea inclusions (Alami-Durante et al., 2010b). Clearly then, plant protein ingredients lead to significant changes in reported muscle fiber cellularity (Alami-Durante et al., 2010a). When replacement of FM with a mix of plant protein ingredients at 75–100 % showed a significantly lower median diameter of white muscle fibers in juvenile rainbow trout (Alami-Durante et al., 2010a), this was suggested to be a consequence of increased cathepsin D expression, an enzyme involved in proteolysis. Furthermore, total replacement of FM with rice protein concentrate (RPC) resulted in a decrease in the large diameter ($> 50\,\mu m$) of white muscle fiber of blunt snout bream (*Megalobrama amblycephala*) (Cai et al., 2018). Changes in muscle fiber growth was used to explain the poor growth performance of fish fed RPC in that experiment. However, diets used in the present study were isoproteic, and balanced with essential amino acids; thus poor growth in fish receiving PP75 is not explained by changes in muscle cellularity.

5. Conclusion

Based on the results of the present study, it can be concluded that lumpfish are capable of utilizing mixtures of plant protein concentrates in their diet. The FM in diets for juvenile lumpfish can be replaced with up to 50 % of SPC and PPC without adversely affecting growth performance, body chemical composition, or fast muscle fiber cellularity.

CRediT authorship contribution statement

Florence Perera Willora: Conceptualization, Formal analysis, Investigation, Writing - original draft, Visualization. **Nimalan Nadanasabesan:** Formal analysis, Investigation. **Helene Rønquist Knutsen:** Formal analysis, Validation, Visualization. **Cui Liu:** Investigation. **Mette Sørensen:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Project administration. **Ørjan Hagen:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2020.100352.

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