Aquaculture Reports 13 (2019) 100175

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Muscle growth and changes in chemical composition of spotted wolffish juveniles (*Anarhichas minor*) fed diets with and without microalgae (*Scenedesmus obliquus*)

H.R. Knutsen[a], O.H. Ottesen[a], A.M. Palihawadana[a], W. Sandaa[b], M. Sørensen[a], Ø. Hagen[a,*]

[a] Nord University, Faculty of Bioscience and Aquaculture, Norway
[b] Aminor AS, 8178, Halsa, Norway

ARTICLE INFO

Keywords:
nutrition
sustainable aquafeed
muscle fiber cellularity
muscle growth
proximate composition
histology
alternative feed ingredients

ABSTRACT

Spotted wolffish (*Anarhichas minor*) is a promising new candidate for cold-water fish farming, but knowledge is needed about its physiology and its capacity to utilize alternative feed ingredients. The aim of the study was to investigate fast muscle growth dynamics, changes in chemical composition as well as growth performance of spotted wolffish when fed diets with or without the microalgae *Scenedesmus obliquus* incorporated. Juvenile spotted wolffish were fed four diets containing fishmeal as the primary source of protein (CTR diet) or microalgae (*Scenedesmus obliquus*) replacing 4% (AL4 diet), 8% (AL8 diet) or 12% (AL12 diet) of the fishmeal. During the 12 week experiment, fish grew from an average weight of 140 g to 250 g. The results showed indications of fast muscle cellularity of spotted wolffish being affected by dietary algae inclusion as the control and AL4 groups appeared to be more strongly favored by hypertrophic growth compared to the AL8 and AL12 groups. The CTR and AL4 groups tended towards increased muscle fiber diameters and higher proportions of larger muscle fibers, while the AL8 and AL12 group tended towards similar or increased proportions of smaller muscle fibers at the end of the trial. Probability density functions showed no difference in fast muscle fiber size distributions between dietary groups. Muscle crude protein and fat content tended to increase with growth in all treatment groups and muscle mineral content was reduced in all groups fed diets containing *Scenedesmus*. At the end of the trial, hepatosomatic index was reduced in all treatment groups. Dietary replacement of fishmeal with *Scenedesmus* also affected skin coloration, with increasing yellowness observed with increasing microalgae replacement. This study indicates that spotted wolffish has the potential to use microalgae as an alternative to fishmeal in the diet.

1. Introduction

Spotted wolffish (*Anarhichas minor*, Olafsen) is a North Atlantic fish species with promising potential for aquaculture production. Good growth performance at low temperatures, tolerance for high stocking density and robustness towards stress and disease make the spotted wolffish favorable for diversification of aquaculture in the North-Atlantic (Foss et al., 2004). The edible portion of fish consists primarily of muscle, which is also the largest tissue mass, comprising 60% or more of the fish body (Sänger and Stroiber, 2001). Fish increase their muscle mass by both hyperplastic and hypertrophic growth. As opposed to other vertebrates such as mammals and birds, fish can recruit new fibers for an extended part of their post-embryonic life. Hypertrophic growth occurs in fish throughout their life until the muscle fibers have reached their maximum diameter. The relative contribution of hyperplasia and hypertrophy to increases in muscle mass can affect the muscle cellularity. Muscle cellularity is affected by a number of factors including temperature (Johnston et al., 2000) and photoperiod (Johnston et al., 2003). Diet has also been shown to influence muscle cellularity (e.g. Alami-Durante et al., Bjørnevik et al., 2003, 2010, Silva et al., 2009). However, to date, little is known regarding the effect of alternative feed ingredients on fish muscle cellularity. The only study to our knowledge is the report by Alami-Durante et al. (2010) investigating how changes in dietary plant protein sources and amino acid profiles affect muscle growth in rainbow trout (*Onchorynchus mykiss*).

Fishmeal has historically provided a cheap and high quality source of dietary protein for both farmed aquatic animals and terrestrial livestock (Olsen and Hasan, 2012, Tacon and Meitan, 2015). However, the wild fish stocks that fishmeal production depend on are being

* Corresponding author.
E-mail addresses: helene.r.knutsen@nord.no (H.R. Knutsen), oddvar.ottesen@nord.no (O.H. Ottesen), anjana.m.palihawadana@nord.no (A.M. Palihawadana), willy@aminor.no (W. Sandaa), mette.sorensen@nord.no (M. Sørensen), orjan.hagen@nord.no (Ø. Hagen).

https://doi.org/10.1016/j.aqrep.2018.11.001
Received 7 June 2018; Received in revised form 18 October 2018; Accepted 6 November 2018

Table 1
Ingredients (g 100 g^{-1} diet) and proximate composition [%] of the experimental diets containing different levels of microalgae (*Scenedesmus obliquus*) as a replacement for fish meal.

	Treatment diet			
	CTR	AL4	AL8	AL12
Ingredients (g 100 g^{-1} diet)				
Scenedesmus obliquus[1]	-	4.00	8.00	12.00
Fish meal LT70[2]	80.00	76.00	72.00	68.00
Fish oil[3]	7.00	7.00	7.00	7.00
Wheat meal[4]	12.30	12.30	12.30	12.30
Micro-ingredients[5]	0.70	0.70	0.70	0.70
Proximate composition of diets (g 100 g^{-1} ± S.E.M)				
Crude fat	16.12 ± 0.03	15.88 ± 0.04	16.01 ± 0.07	16.37 ± 0.28
Crude protein	52.12 ± 0.22	52.33 ± 0.30	52.91 ± 0.06	51.92 ± 0.45
Ash	13.48 ± 0.41	13.30 ± 0.37	12.20 ± 0.20	12.17 ± 0.20
Water	8.97 ± 0.07	9.50 ± 0.05	8.56 ± 0.05	8.29 ± 0.05
Energy (KJ g^{-1})	20.61	20.69	21.01	21.10
Amino acid composition (g 100 g^{-1})				
Asparagine	4.99	4.65	4.73	4.70
Serine	2.28	2.13	2.30	2.30
Glutamic acid	7.08	7.02	7.44	7.67
Proline	2.51	2.71	2.79	2.90
Glycine	3.98	3.84	3.88	3.91
Alanine	3.44	3.35	3.42	3.46
Valine	2.56	2.49	2.56	2.59
Isoleucine	2.14	2.03	2.05	2.08
Leucine	3.85	3.67	3.76	3.82
Tyrosine	1.72	1.60	1.66	1.67
Phenylalanine	2.18	2.00	2.11	2.25
Histidine	1.12	1.06	1.08	1.08
Lysine	4.02	3.76	3.74	3.68
Arginine	3.12	3.02	2.95	2.97
Tryptophan	0.584	0.558	0.580	0.604
Cysteine	0.466	0.442	0.464	0.502
Methionine	1.62	1.49	1.51	0.824
Hydroxyproline	0.676	0.457	0.610	0.561
Ornitine	< 0.05	< 0.05	< 0.05	< 0.05
Treonine	2.32	2.14	2.26	2.24

Treatment diets: CTR: control. AL4: 4% Scenedesmus inclusion. AL8: 8% Scenedesmus inclusion. AL12: 12% Scenedesmus inclusion.
1 Protein: 45.7%; lipid: 9.1%; carbohydrates: 15.6%; dietary fiber: 15.8%; ash: 8.3%; moisture: 5.6%; energy: 1.5 MJ g-1; pigments: 2.056% chlorophyll, 0.607% total carotenoids (Allma, Lisbon, Portugal).
2 Protein: 70%; lipid: 5.8% (Sopropeche, France)
3 SAVINOR UTS, Portugal
4 Protein: 11.7%; lipid: 1.6% (Casa Lanchinha, Portugal).
5 Vitamin & Mineral Premixi: 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.

depleted. As such, the supply of fishmeal cannot keep pace with the demand resulting in a tripling of the cost of fishmeal since the early 2000's. Fishmeal is increasingly becoming both environmentally and economically unsustainable (FAO, 2016). Therefore, fishmeal as a dietary protein source cannot supply the world's growing aquaculture industry alone. To reduce the industry's dependence on fishmeal and fish oil, evaluation of novel feed ingredients is necessary.

Microalgae are potential feed ingredients in aquacultural diets. Some strains of microalgae contain important nutrients, including protein with a balanced amino acid profile, polyunsaturated fatty acids, vitamins and health promoting compounds (Becker, 2007, López et al., 2010, Yaakob et al., 2014). An increasing number of reports demonstrate that microalgae can partially replace fishmeal for several commercially important species of cultured fish. For example, moderate levels (10%) of the microalgae *Nannochloropsis oceanica* could be included in the diet of Atlantic salmon (*Salmo salar*) without adverse effects on fish performance (Sørensen et al., 2017). Other species of microalgae incorporated in diets for Atlantic salmon also showed positive effects on fillet quality and retention of essential fatty acids (Kiron et al., 2012, Kousolaki et al., 2016, Kousolaki et al., 2015). *Tetraselmis suecica* and *Isochrysis* sp. could replace 20% of the fishmeal in European seabass (*Dicentrarchus labrax*) diets without adverse effects on performance (Tibaldi et al., 2015, Tulli et al., 2012). Replacement of fishmeal with microalgae has also been successful for other cultured fish species such as sea bream (*Sparus aurata*), red drum (*Sciaenops ocellatus*) as well as for terrestrial livestock (Patterson and Gatlin, 2013, Vizcaíno et al., 2014, Yaakob et al., 2014). The freshwater green algae *Scenedesmus obliquus* may be a potential ingredient for marine fish. *S. obliquus* has a relatively high protein content (50-56% of dry matter) and contains all essential amino acids as reported by Becker (2007). The closely related *Scenedesmus almeriensis* significantly increased intestinal absorptive capacity and could replace up to 20% of the fishmeal in the diet of gilthead sea bream (*Sparus aurata*) without adverse effects on fish performance (Vizcaíno et al., 2014). Bawdy et al. (2008) reported that *Scenedesmus* sp. could replace up to 50% of fish meal in Nile tilapia (*Oreochromis niloticus*) diets. To date, some of the largest challenges in using microalgae as feed ingredients in aqua diets are high production cost and low available volumes (Benemann, 2013). One of the advantages of *S. obliquus* is the possibility for large scale production (Basu et al., 2014, Becker, 2007).

Replacement of fishmeal with alternative feed ingredients for spotted wolfish has not yet been investigated. The aim of the present

study was to investigate the effect of partial replacement of fishmeal with *S. obliquus* as a source of dietary protein on (i) fast muscle growth dynamics, (ii) muscle proximate composition as well as (iii) overall biometric gain and somatic indexes.

2. Materials and methods

The experiment was carried out at Mørkvedbukta research station at Nord University (Bodø, Norway) following the Guidelines of the European Union (Directive 2010/63/UE) for the use of laboratory animals. The study was approved by the Animal Welfare Committee at FBA, Nord University and carried out in strict accordance with the Norwegian animal welfare act (LOV-2009-06-19-97) and the regulation on the use of animals in research (FOR-2015-06-18-761). Animal sacrifice was limited to the minimum required to conduct the trial.

2.1. Fish rearing facility and husbandry

Juvenile spotted wolffish (mean weight 60 g) was provided by Aminor AS (Halsa, Norway). Fish were randomly distributed into 12 circular tanks (1 m^3) with $n = 75$ fish per tank and acclimatized to the laboratory conditions for 7 weeks. During acclimatization, fish were fed a commercial diet (Amber Neptun, Skretting, Stavanger, Norway). The fish were adapted to the experimental diets over a 15 day period, starting with a mix of experimental/commercial feed in the ratio 1/3 for 5 days, 2/3 for 5 days and finally experimental diets only for 5 days. Fish were provided seawater from a flow through treatment system. Filtered (200 µm) and aerated seawater with stable salinity (34 ‰), temperature (7.7 °C ± 0.005) and oxygen (86.7 ± 0.11 %) was supplied from 250 m depth in Saltfjorden. Water flow was at 1400 L/h throughout the trial. Fish were kept under continuous light and with continuous meal-based feeding (in excess) between 08:00 and 21:00 during the experiment. Feeding rate was set to 1.6% and gradually reduced to 1.4% of total biomass towards the end of the trial based on appetite and accumulated feed waste.

2.2. Experimental diets and feeding trial

Feed ingredients and proximate composition of the experimental diets are shown in Table 1. Four diets were formulated to contain 0% (CTR), 4% (AL4), 8% (AL8) and 12% (AL12) of microalgae substituting fishmeal on an equal weight basis. Diets were otherwise identical in composition, as they were balanced based on crude chemical composition and formulated to be isonitrogenous, isolipidic and isocaloric. The microalgae (*Scenedesmus obliquus*) was cultured at Allma (Lisbon, Portugal) in closed photobioreactors and spray dried prior to inclusion in the diets. The experimental diets were extruded with a pellet diameter of 2-3 mm produced at Sparos Lda. (Olhão, Portugal). Diets were stored at room temperature, in air-tight containers and protected from light during the trial. Following the acclimatization period of the fish, the four diets were randomly allocated to triplicate tanks. At the start of the experiment fish were about 1 year old, with a body weight of 139.87 ± 1.08 g and length of 22.70 ± 0.05 cm (mean ± SEM). The feeding experiment was terminated after 12 weeks.

2.3. Sampling

Fish (n = 10 per tank, altogether n = 120 fish) were randomly sampled with four week intervals: at week 0, 4, 8 and 12 of the trial. The fish were euthanized with an overdose of the anaesthetic MS-222 (tricaine methanesulfonate, 0.14 g/L) buffered with equal parts sodium bicarbonate followed by mild cranial concussion. Weight, total length and liver weight was individually recorded for all sampled fish. Weight of fish was recorded to the nearest 0.5 g, total length to the nearest 0.1 mm and liver weight to the nearest 0.01 g. At week 0 and 12 all fish were anaesthetized with a non-lethal dose of buffered MS-222 (0.0875 g/L) prior to weighing and length measurements. At termination of the experiment (week 12), skin color was measured using a portable spectrophotometer (CM-700d, Konica Minolta Sensing Inc., Singapore). At each sampling point, individual fillets were taken for analysis of proximate biochemical composition and samples were frozen at −40 °C until further analysis. From four of the euthanized fish per tank, samples were taken for histological analysis of muscle (see section 2.4). Feed samples were collected at the beginning of the experiment and stored at −40 °C for analysis of proximate chemical composition and energy content.

2.4. Proximate composition

All proximate composition analyses were performed in duplicates. Muscle samples were thawed, pooled (n = 3 fish per tank) and analyzed for crude protein, ash, moisture and crude fat content. Moisture content was determined by drying samples (5 g) to a constant weight (20 hours at 105 °C) in a drying cabinet. Ash (mineral) content was obtained by burning samples (5 g) in a crucible in a muffle furnace (16 hours at 540 °C). Crude protein content was determined using the Kjeldahl titration method. Samples (1 g) were weighed out and hydrolyzed for 45 min at 420 °C with 15 mL sulfuric acid (H_2SO_4, 98%, VWR chemicals) and Kjeldahl catalyst tablets (3.5 kg K_2SO_4 and 0.4 g $CuSO_4$, Foss, Sweden). Room temperature samples were diluted in distilled water (75 mL) were then analyzed in a Kjeldahl titrator (Kjeltec™ 2300, Foss, Sweden). Crude fat content was determined by the diethyl ester extraction method. Samples (10 g) were measured and water free sodium sulfate (20 g, Na_2SO_4, VWR chemicals) was added and mixed to a dry powder. Ethyl acetate (50 mL, $C_4H_8O_2$, VWR chemicals) was added to the samples and stirred for an hour. The solution was then filtered and the solution (20 mL) was placed in steam bath for 15 min in an evaporation cup to remove the solvent. After evaporation of the solvent, the evaporation cup was dried at 105 °C for 15-20 min and cooled in a desiccator. The proximate composition of the feed was determined using the same methods as for the muscle samples. Gross energy was determined using a bomb calorimeter (IKA C200 bomb calorimeter, Staufen, Germany).

2.5. Fast muscle cellularity

Myotomal steaks (5 mm thickness) were cut anterior to the posterior ventral fin. A photograph of the steak was taken together with a scale. The total cross-section area (TCA) was then measured in these images using the software ImageJ (NIH, USA). Blocks (n = 2-4 depending on fish size, 5 × 5 x 5 mm) of fast muscle were prepared from each steak from the left side of the body to cover the steak area. Muscle blocks were mounted on small pieces of cork sheet (1.5 x 1.5 cm), covered in Cryomatrix (Shandon Cryomatrix, Thermo Scientific) and cooled in liquid nitrogen for 45 s in 2-methyl butane (isopentane, VWR chemicals) to near the freezing point (−159 °C). Frozen blocks were wrapped in pre-labeled tinfoil and stored at −80 °C. Prior to sectioning, blocks were acclimated to −20 °C for 30 min. Sections (7 µm) were prepared using a cryostat (Cryostar NX50, Thermo Scientific), mounted and air-dried on poly-L-lysine coated slides and stained with Hematoxylin (Meck Chemicals) for 12 min. Sections were then rinsed for 10 min under running tap water and a cover-glass was mounted on the section using glycergel (Glycergel mounting medium, Dako). Area of muscle fibers were measured on images at 10x magnification using a microscope (Axioskop2, Carl Zeiss) and camera (Axiocam HRC, Carl Zeiss) using the Carl Zeiss software, AxioVision 4.8. An illustrative photo of a muscle cross section is shown in Fig. 1. A minimum of 800 fibers were measured in total per fish.

2.6. Calculations

Weight gain (WG, %) was calculated from WG = ((final mean

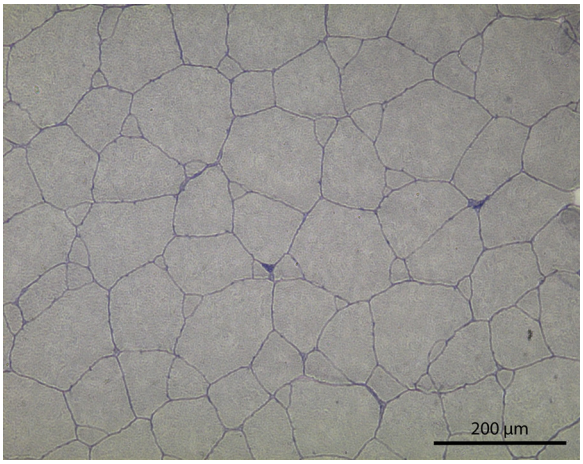

Fig. 1. Cross section (10x) of fast skeletal muscle of spotted wolffish showing smaller newly recruited fibers scattered among older larger fibers typical for the mosaic hyperplastic growth phase in juvenile fish.
Color online only

weight – initial weight)/initial weight) * 100. Specific growth rate (SGR, % day^{-1}) was calculated as 100 x ln[final mean weight (g) / initial mean weight (g)] / days. Condition factor (CF) was calculated as [fish weight (g) / total length $(cm)^3$] x 100. Hepatosomatic index (HSI) was calculated as [liver weight (g) / fish weight (g)] x 100.

Percent moisture was calculated according to the following formula: Moisture (%) = ((WW-(DW-ECW))/WW) x 100%, where WW is the sample wet weight, DW is the sample dry weight and ECW is the empty cup weight for the cup used during drying. Percent ash was calculated according to the following formula: Ash (%) = ((FCW-ECW)/SW) x 100, where FCW is the fired crucible weight, ECW is the empty crucible weight and SW is sample weight. Fat (g) was calculated according to the following formula: Fat (g) = ECF – ECE, where ECF is the weight of the evaporation cup with fat and ECE is the empty evaporation cup. Percent Fat was calculated according to the following formula: Fat (%) = (10,300 x F)/(40 – 2.17 x F x SW), where 10,300 is a constant and 40-2.17 is a calibration factor, F is the fat content (g) and SW is the sample weight.

Diameter and number of muscle fibers was calculated according to Johnston et al. (1999). Diameter of muscle fibers were calculated from the measured area using the formula 2 x (Square root(Muscle fiber area/π)).. Muscle fiber number (FN) was calculated from the formula FN = 10^6 x (TCA x number of counted fibers / sum area). Muscle fiber density (FD) was calculated from the formula FD = 10^6 x (number of counted fibers / sum area). Fiber recruitment per day was calculated from the formula (FN_1 – FN_0)/days, where FN_0 is the group-mean muscle fiber number at week 0 and FN_1 is the group-mean muscle fiber number at the end of the experiment. As fast muscle cellularity is dependent on fish size (Weatherly et al., 1988) the data was also normalized based on fish size according to the normalization reported by Alami-Durante et al (2010). The parameters that were increasing with increasing standard length were divided by the natural logarithm of the standard length (ln SL). Parameters that were decreasing with increasing standard length were multiplied by ln SL. All data are presented as mean ± SEM.

2.7. Statistical analysis

All data were tested for normality by the Shapiro-Wilk test and homogeneity of variance by Levene's mean test before being analyzed with a one-way ANOVA using the software Sigmaplot 12.0 (Systat Software, San Jose, CA). When the ANOVA showed significant differences, the Holm-Sidak method of multiple comparisons was used to compare individual means. When the data did not meet the ANOVA assumptions, a Kruskal-Wallis one-way analysis of variance on ranks was used. When the Kruskal-Wallis test showed significance, Dunn's method of multiple comparisons was used to compare individual medians. Means of replicate tanks were also compared with a one-way ANOVA.

To compare the distribution of muscle fiber sizes, nonparametric statistical techniques were used to fit smoothed probability density functions (pdfs) to the measurements using a kernel function (Bowman and Azzalini, 1997) as described by Johnston et al. 1999. This was done using the software R-3.4.1 (R Core Team, 2017) and package sm 2.2-5.4 (Bowman and Azzalini, 2014). Altogether, six fish of equal total length (28.0 ± 1.0 cm) were selected from each treatment group and smoothed pdfs were fitted to the fish in each group as well as the group mean. Bootstrap techniques were then used to plot approximate variability bands around the group pdfs using the mean smoothing parameter. This provided a visual indication of which areas of the muscle fiber distribution that was potentially significantly different. In addition, a Kolmogorov-Smirnov two-sample test was used to test if the pdf of the treatment groups were equal over all diameters. In all tests, significant differences were considered when $p \leq 0.05$.

3. Results

3.1. Biometrical data

Biometrical data is presented in Table 2. No mortality was noted during the trial and the fish weight was nearly doubled for most groups. However, at termination of the experiment, fish fed the AL8 diet had lower mean weight, total length, %WG and SGR compared to all other dietary groups. No differences in mean weight, length, %WG and SGR was observed among the other treatment groups ($p > 0.05$). At week 12 the AL8 and AL12 had lower condition factor compared to both the start of the experiment ($p < 0.001$) and to the control group ($p < 0.001$ and $p = 0.001$ respectively).

Mean hepatosomatic index (mean ± SEM) at the start of the experiment was 4.76 ± 0.04. Interestingly, all groups had lower hepatosomatic index at week 12 compared to week 0, but only the AL8 group (3.64 ± 0.07) was different from the control (3.82 ± 0.07) at week 12 ($p = 0.003$). The hepatosomatic index was lower for all groups already from week 4 ($p < 0.05$). The AL8 group was also the only group that showed no increase in mean liver weight at week 12 compared to week 0 (not shown in table, $p > 0.05$).

3.2. Fast muscle cellularity

Fast muscle cellularity is shown in Table 3. The results showed no difference ($p > 0.05$) in fast muscle cellularity between the different treatment groups for any of the assessed parameters at the end of the trial. Differences were observed when comparing the treatment groups to the means at the start of the trial. All treatments had increased fiber number at week 12, but AL12 was the only group that was different from week 0 ($p = 0.023$). Reduced fiber density at week 12 was also observed in all groups. The D mean and D median was increased for all groups at week 12, but D mean was only different for the CTR and AL4 group ($p = 0.027$ and $p = 0.007$ for AL4 and CTR respectively) and D median only for the AL4 group ($p = 0.011$). The CTR group was also the only group that showed increased D max ($p = 0.018$) at week 12. D mean of the upper 95 percentile was also increased in all groups ($p = 0.001$, 0.010 and 0.028 for the CTR, AL12 and Al4 groups respectively) with the exception of AL8 that was not different from week 0 ($p > 0.05$). Compared to the start of the trial, the proportion of fast muscle fibers with diameter ≤ 20 µm was decreasing in all treatment groups, but was only significant for the CTR group ($p = 0.035$). In all groups, there was no difference in proportion of fibers with $20 < D \leq 40$ µm and $80 < D \leq 120$ µm. Proportion of fibers in the range $40 < D \leq 80$ µm was decreasing in all groups, but was lower

Table 2
Survival, weight, length, condition factor (CF), hepatosomatic index (HSI), total weight gain (WG) and daily growth (DG) of spotted wolffish fed diets with different level of inclusion of microalgae (*Scenedesmus obliquus*). Weight, length, WG, SGR and CF for week 0 and week 12 are based on measurements of all fish. All other values are based on sampled fish. Values are means ± SEM. Means in the same column at the same time point with different superscript letters differ significantly ($P < 0.05$). Means in the same column with superscript * differ significantly from the start of the trial ($P < 0.05$).

Time	Diet	Survival [%]	Body weight [g]	Body length [cm]	CF	HSI	WG [%]	SGR [% day^{-1}]
Week 0	Start	n.a	139.87 ± 1.08	22.70 ± 0.05	1.18 ± 0.004	4.76 ± 0.04	n.a	n.a
Week 4	CTR	100	194.88 ± 6.74^{*a}	25.45 ± 0.26^{*a}	1.17 ± 0.01^{a}	4.21 ± 0.05^{*a}	39.11 ± 3.84^{a}	1.06 ± 0.09^{a}
	AL4	100	182.13 ± 6.18^{*ab}	24.86 ± 0.27^{*ab}	1.17 ± 0.02^{a}	4.02 ± 0.05^{*ab}	29.31 ± 6.58^{a}	0.82 ± 0.17^{a}
	AL8	100	164.47 ± 7.22^{*b}	24.23 ± 0.28^{*b}	1.14 ± 0.02^{a}	3.98 ± 0.06^{*b}	18.22 ± 1.12^{a}	0.54 ± 0.03^{a}
	AL12	100	181.47 ± 7.52^{*ab}	25.09 ± 0.31^{*ab}	1.13 ± 0.02^{a}	3.94 ± 0.07^{*b}	30.20 ± 3.75^{a}	0.84 ± 0.09^{a}
Week 8	CTR	100	231.95 ± 11.69^{*a}	27.03 ± 0.38^{*a}	1.15 ± 0.01^{a}	3.85 ± 0.07^{*ab}	65.70 ± 3.45^{a}	0.87 ± 0.04^{a}
	AL4	100	237.98 ± 10.85^{*a}	27.12 ± 0.34^{*a}	1.17 ± 0.02^{a}	4.06 ± 0.08^{*a}	68.55 ± 9.41^{a}	0.89 ± 0.10^{a}
	AL8	100	217.98 ± 10.01^{*a}	26.66 ± 0.35^{*a}	1.13 ± 0.02^{a}	3.64 ± 0.08^{*b}	56.70 ± 6.42^{a}	0.77 ± 0.07^{a}
	AL12	100	230.23 ± 7.91^{*a}	26.84 ± 0.30^{*a}	1.18 ± 0.02^{a}	3.75 ± 0.06^{*b}	65.70 ± 3.45^{a}	0.86 ± 0.04^{a}
Week 12	CTR	100	262.63 ± 5.56^{*a}	27.98 ± 0.18^{*a}	1.18 ± 0.01^{a}	3.82 ± 0.07^{*a}	87.63 ± 1.33^{a}	0.72^{a} ± 0.01^{a}
	AL4	100	265.34 ± 6.44^{*a}	28.13 ± 0.19^{*a}	1.16 ± 0.01^{ab}	3.73 ± 0.06^{*a}	88.20 ± 4.55^{a}	0.73^{a} ± 0.03^{a}
	AL8	100	221.59 ± 4.85^{*b}	26.94 ± 0.17^{*b}	1.11 ± 0.01^{*c}	3.64 ± 0.07^{*b}	59.31 ± 3.19^{b}	0.53^{b} ± 0.02^{b}
	AL12	100	247.10 ± 5.73^{*a}	27.72 ± 0.18^{*a}	1.13 ± 0.01^{*bc}	3.74 ± 0.07^{*a}	77.25 ± 2.26^{a}	0.66^{a} ± 0.01^{a}

only for the AL4 and CTR groups ($p = 0.005$ and 0.018 for AL4 and CTR respectively). The AL8 group was the only group that did not show any increase in number of fibers in the range $120 < D > 200$ µm.

Number of fibers recruited per day increased with increasing algae replacement. Fiber recruitment was 471, 322, 174 and 48 fibers day^{-1} for the AL12, AL8, AL4 and CTR groups, respectively. The probability density functions for muscle fiber size distributions showed no differences between the treatment groups (Fig. 2).

3.3. Proximate composition of muscle

The muscle proximate composition is shown in Table 4. Except for crude protein, there was no difference in muscle proximate composition among treatment groups at the end of the experiment ($p > 0.05$). Compared to the start of the experiment, muscle crude protein content and fat content tended to be slightly increased for all groups (Table 4). The spotted wolffish also had a subcutaneous fat layer (Fig. 3). At termination of the experiment, compared to week 0, all algae treatment groups had reduced muscle mineral content ($p = 0.007$, $p < 0.001$, $p = 0.004$ for AL4, AL8 and AL12 respectively) and the CTR and AL4 had reduced muscle moisture ($p < 0.001$ and $p = 0.008$ respectively).

3.4. Skin color

No difference was observed between treatments for skin color L* value (lightness, Fig. 4A, $p = 0.566$) and a* value (red/green, Fig. 4B, $p = 0.414$). However, the skin color b* value differed significantly among feeding groups (blue/yellow, Fig. 4C, $p < 0.001$), where the skin of the fish was increasingly more yellow with increasing algae inclusion of the diet. AL12 had higher b* value than AL4 ($p = 0.002$). Although not measured, no visual change in fillet color was observed.

4. Discussion

Overall, the fish performed well during the current experiment, displaying similar or even better growth performance compared to previously published data from juvenile spotted wolffish. The SGR calculated at termination of the trial varied between 0.53 and 0.73% for the different treatment groups and was in accordance with other reports of spotted wolffish. Falk-Petersen et al. (1999) reported that specific growth rates of 150-700 g (1-2 year old) wolffish ranged between 0.37-0.50% day^{-1}. Tremblay-Bourgeois (2010) also reported SGR of 0.65% day^{-1} for 160 g juvenile wolffish reared at optimal density. Imsland et al. (2006) found an inverse relationship between size and growth rate for spotted wolfish, a tendency also observed in the present trial. The lower growth of the AL8 treatment group contradicts findings in other

Table 3
Fast muscle cellularity of spotted wolffish fed diets with different levels of inclusion of microalgae (*Scenedesmus* sp.). Values are means ± SEM. Means in the same row with different superscript letters differ significantly ($P < 0.05$). Significance is presented from analysis of data normalized by total length (TL). For this normalization, parameters increasing with increasing TL were divided by ln TL and parameters decreasing with increasing TL were multiplied by ln TL.

Time	Week 0	Week 12			
	Start	CTR	AL4	AL8	AL12
Fiber number	125834 ± 3865^{a}	145866 ± 4634^{ab}	140651 ± 7492^{ab}	150185 ± 6096^{ab}	155686 ± 7065^{b}
Fiber density [fibers mm^{-2}]	188.02 ± 5.17^{a}	139.10 ± 9.46^{b}	138.24 ± 8.29^{b}	158.75 ± 7.96^{b}	144.03 ± 7.60^{b}
D mean	75.47 ± 1.18^{a}	87.36 ± 2.56^{b}	88.44 ± 2.84^{b}	81.53 ± 2.03^{ab}	85.89 ± 2.62^{ab}
D median	73.34 ± 1.41^{a}	85.59 ± 3.38^{ab}	87.59 ± 3.18^{b}	79.63 ± 2.52^{ab}	84.42 ± 2.88^{ab}
D max	194.70 ± 2.51^{a}	227.73 ± 6.92^{b}	227.05 ± 10.31^{ab}	218.28 ± 9.20^{ab}	224.92 ± 7.91^{ab}
D mean of upper 95th percentile	156.58 ± 1.78^{a}	182.81 ± 4.73^{b}	176.40 ± 4.29^{b}	170.37 ± 4.74^{ab}	179.21 ± 4.39^{b}
Proportion [%] of white muscle fibers with					
$D \leq 20$ µm	4.28 ± 0.33^{a}	2.47 ± 0.30^{b}	2.82 ± 0.48^{ab}	3.30 ± 0.63^{ab}	2.94 ± 0.49^{ab}
$20 < D \leq 40$ µm	14.43 ± 0.84^{a}	13.91 ± 1.07^{a}	12.31 ± 1.17^{a}	13.87 ± 1.08^{a}	12.83 ± 1.42^{a}
$40 < D \leq 80$ µm	38.55 ± 1.35^{a}	29.02 ± 2.65^{b}	27.67 ± 1.72^{b}	33.78 ± 2.61^{ab}	30.79 ± 1.17^{ab}
$80 < D \leq 120$ µm	30.70 ± 1.03^{a}	32.20 ± 1.84^{a}	34.85 ± 1.15^{a}	32.00 ± 1.30^{a}	33.54 ± 1.00^{a}
$120 < D \leq 160$ µm	10.16 ± 0.52^{a}	16.13 ± 1.34^{b}	17.36 ± 1.59^{b}	13.32 ± 1.30^{ab}	14.39 ± 1.29^{b}
$160 < D \leq 200$ µm	1.77 ± 0.20^{a}	5.29 ± 0.82^{b}	4.37 ± 0.74^{b}	3.28 ± 0.60^{ab}	4.85 ± 0.74^{b}
$D > 200$ µm	0.11 ± 0.04^{a}	0.98 ± 0.25^{b}	0.62 ± 0.19^{b}	0.45 ± 0.18^{ab}	0.66 ± 0.20^{b}

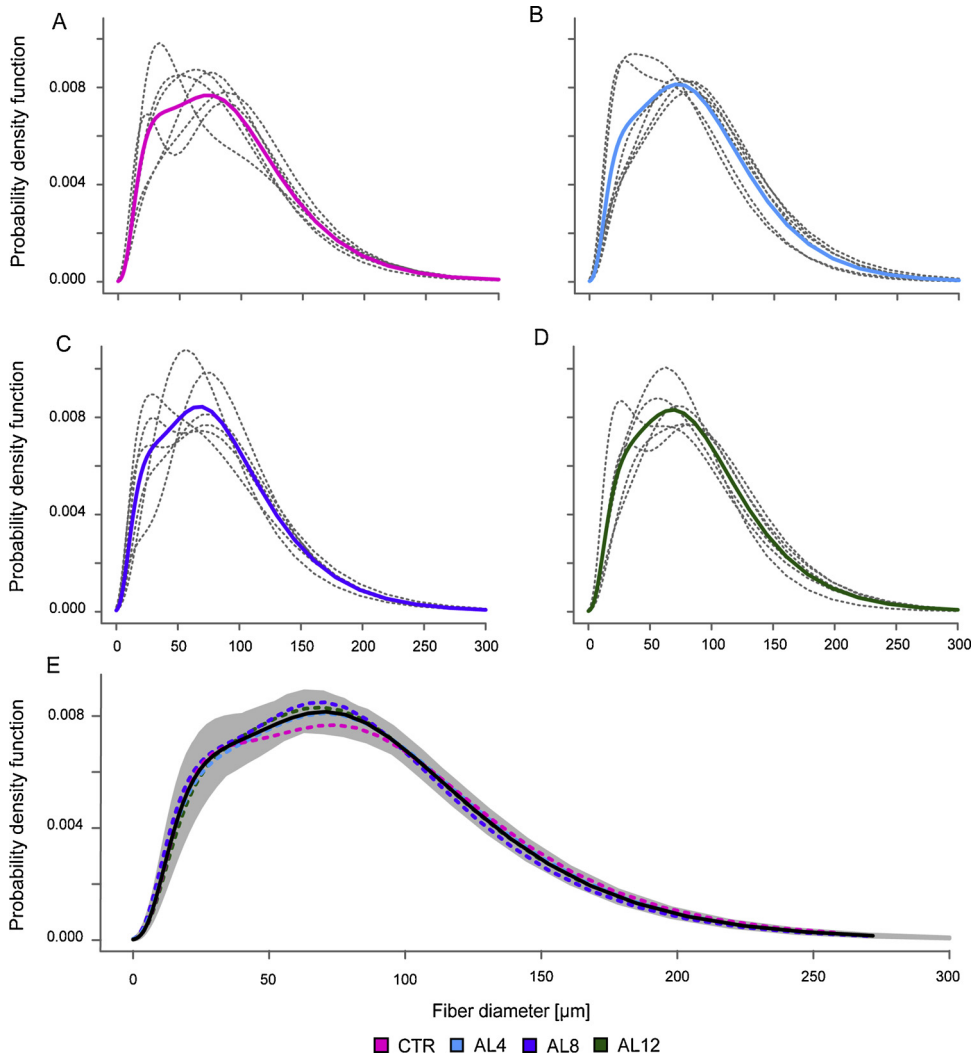

Fig. 2. Muscle fiber diameter probability density functions of *A. minor* fed increasing levels of *S. obliquus* meal in the diet. For A-D solid line is group mean and dotted line is individual fish. A: Control treatment. B: Treatment group AL4. C: Treatment group AL8. D: Treatment group AL12. E: Bootstrapping analysis comparing the four treatment groups over all fast fiber diameters using Kolmogorov-Smirnov statistics.
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Table 4
Muscle proximate composition [%] of spotted wolffish fed diets with different level of inclusion of microalgae (*Scenedesmus obliquus*). Values are means ± SEM. Means in the same column at the same time point with different superscript letters differ significantly ($P < 0.05$). Means in the same column with superscript * differ significantly from the start of the trial ($P < 0.05$).

Time	Diet	Crude protein	Crude lipid	Ash	Moisture
Week 0	Start	17.10 ± 0.07	4.15 ± 0.16	1.29 ± 0.01	76.95 ± 0.14
Week 4	CTR	17.35 ± 0.22^{a}	4.19 ± 0.32^{a}	1.32 ± 0.03^{a}	77.11 ± 0.28^{a}
	AL4	17.58 ± 0.17^{a}	3.32 ± 0.26^{a}	1.25 ± 0.02^{a}	77.43 ± 0.17^{a}
	AL8	17.43 ± 0.10^{a}	3.67 ± 0.22^{a}	1.29 ± 0.01^{a}	77.51 ± 0.24^{a}
	AL12	17.65 ± 0.15*^{a}	3.84 ± 0.26^{a}	1.26 ± 0.01^{a}	77.14 ± 0.20^{a}
Week 8	CTR	17.82 ± 0.08*^{a}	4.03 ± 0.33^{abc}	1.25 ± 0.02^{b}	77.33 ± 0.35^{a}
	AL4	17.72 ± 0.12*^{a}	4.47 ± 0.28^{b}	1.19 ± 0.02*^{a}	76.59 ± 0.24^{a}
	AL8	17.88 ± 0.14*^{a}	3.36 ± 0.27^{c}	1.26 ± 0.01^{b}	77.48 ± 0.24^{a}
	AL12	17.79 ± 0.12*^{a}	3.71 ± 0.21^{abc}	1.22 ± 0.01*^{ab}	77.24 ± 0.16^{a}
Week 12	CTR	17.51 ± 0.27^{abc}	5.72 ± 0.46*^{a}	1.24 ± 0.02^{a}	75.14 ± 0.50*^{a}
	AL4	18.00 ± 0.15*^{b}	4.94 ± 0.17^{a}	1.22 ± 0.01^{a}*	75.73 ± 0.33*^{a}
	AL8	17.71 ± 0.18*^{abc}	4.66 ± 028^{a}	1.20 ± 0.01^{a}*	76.29 ± 0.23^{a}
	AL12	17.25 ± 0.07^{a}	5.03 ± 0.38^{a}	1.21 ± 0.02^{a}*	76.28 ± 0.35^{a}

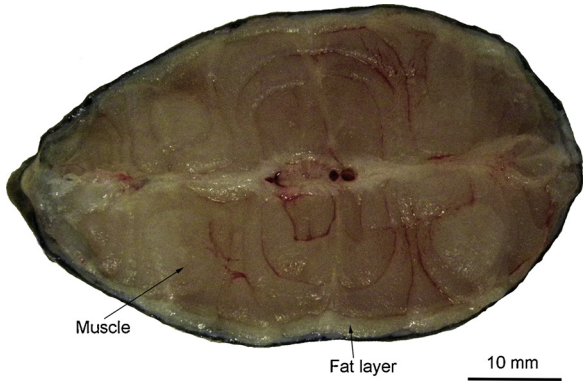

Fig. 3. Cross-section (cut anterior to the posterior ventral fin) showing the subcutaneous fat layer of juvenile *A. minor*.
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studies. Usually lowest growth is found at the highest inclusion levels (e.g. Bawdy et al., 2008, Patterson and Gatlin, 2013, Sørensen et al., 2017, Vizcaíno et al., 2014). This also conflicts with other trials using *Scenedesmus* sp. where no negative effects on growth performance were observed (Bawdy et al., 2008, Vizcaíno et al., 2014). This unexpected result may be explained by larger variation (mean weights 205, 221 and 239 g) among tank means at week 12, resulting in significant differences in among-tank means ($p = 0.027$). However, no tank effects were noted for any other study parameters.

The probability density functions showed no indication that diet influenced the distribution of fast muscle fibers. However, a few significant differences were observed when comparing the individual changes from the start to the end of the trialSpotted wolffish can reach a maximum body length of 180 cm and the fish used in this experiment had a final mean standard length of about 27 cm. Recruitment of new fibers cease at a size of about 44% of the final body length in several teleost fish (Weatherly et al., 1988). If this is true also for spotted wolffish, they are expected to continue increasing muscle mass through hyperplastic growth. Recruitment fibers (fibers ≤ 20 µm) were present in all treatment groups, but tended to be reduced compared to the start of the experiment. However, there seemed to be a correlation between the rate of hyperplasia and algae inclusion. Reduced recruitment was found at the end of the trial for all groups, but it was only significant for the control group. The fast muscle fibers of the control and AL4 groups had in general larger diameter compared to the other groups. In addition, larger fibers appeared to compose an increasing proportion of the total muscle fiber distribution, indicating a favoring of hypertrophic growth. The population of muscle fibers in the AL8 and AL12 groups consisted in general of fibers with smaller diameter; in addition, there was a similar size distribution compared to the start of the trial. This may indicate minimal changes in the ratio between hyperplastic and hypertrophic growth. Increased fiber number at the end of the trial was noted only for the AL12 group. Consequently, the results indicated an increased muscle fiber recruitment as an effect of algae inclusion. The inverse relationship between algae inclusion and daily fiber recruitment also further supports this. Although only significant for the AL8 treatment, both fish fed the AL8 and AL12 diets had numerically lower mean weight at the end of the trial compared to fish fed the AL4 and CTR diets. The favoring of hyperplasia with the higher algae inclusions was most likely associated with the growth dynamics, as fish white muscle growth is influenced both ration level (Kiessling et al., 1991) and dietary composition (Silva et al., 2009; Alami-Durante et al., 2010). Kiessling et al. (1991) also reported a favoring of muscle hypertrophy for rainbow trout in periods of rapid fish growth and a corresponding favoring of muscle hyperplasia in periods of slow growth. At 75-100% substitution of fishmeal with plant protein for juvenile rainbow trout, Alami-Durante et al. (2010) observed a reduced median diameter of white muscle fibers and explained the observation with reduced growth performance and increased expression of cathepsin D, an enzyme involved in lysosomal proteolysis in muscle. The diets used in the present experiment with wolfish were nearly identical in composition, suggesting that more research is needed to explain the mechanism as well as the long-term effects of microalgae on fish growth and performance. However, the probability density functions are much stronger statistical tools for studying muscle fiber populations compared to individual measurements (Johnston, 1999). The results from this analysis should hence be weighted stronger compared to the individual measurements and further studies are necessary to confirm this hypothesis.

Existing reports on the effect of algae inclusion in fish diets report conflicting findings of muscle chemical composition (Bawdy et al. 2008, Dallaire et al., 2007, Nandeesha et al., 2001, Patterson and Gatlin, 2013, Sørensen et al. 2017, Vizcaíno et al. 2014). Vizcaíno et al. (2014) reported no change in muscle proximate composition for sea bream fed *Scenedesmus* in the diet. Bawdy et al. (2008) reported higher dry matter and protein content, but lower lipid content, in the carcass of Nile tilapia fed 50% *Scenedesmus* in the diet. With an intramuscular fat content found to be around 5%, the present juvenile spotted wolffish are classified as intermediately fatty fish (Hocquette et al., 2010). Fat is deposited in the muscle, liver and in the subcutaneous fat layer (shown in Fig. 3). The control treatment was the only treatment with significantly higher intramuscular fat at the end of the trial. It could be hypothesized that the observed tendency for favoring hypertrophic muscle growth in the control group could be connected to lipids being stored in the muscle rather than being metabolized for muscle fiber recruitment. As intramuscular fat positively affects the flavor and quality of fish fillets, increased intramuscular fat could affect the organoleptic properties, and hence the quality of the final product (Hocquette et al., 2010). The significantly higher muscle lipid content in fish fed control diet at the end of the experiment could also indicate higher digestibility and utilization of nutrients and energy compared to

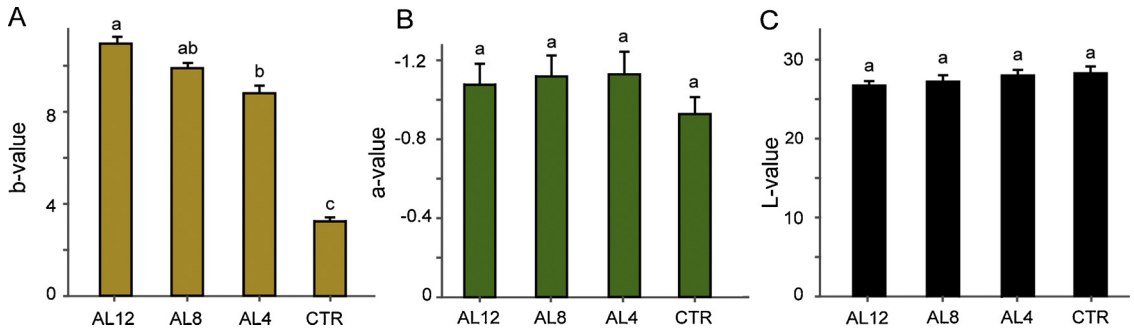

Fig. 4. Spectrophotometric measurements of skin color differences at week 12. Values are means ± SEM. Means with different superscript letters differ significantly *($P < 0.05$)*. A: b-value (blue-yellow, > 0: yellow, < 0: blue. B: a-value (green-red, < 0: green, > 0: red). C L-value (lightness, L = 100: white, L = 0: black).
Color online only.

the algae-fed fish. The higher muscle lipid content may also be explained by the slightly higher body weight of fish fed the control diet. This is in line with Moksness et al. (1995), who reported increased muscle lipid content in groups of common wolffish (*Anachichas lupus*) with the highest growth rate. Moksness et al. (1995) reported no differences in muscle crude protein of common wolffish with different growth rate. Compared to the start of the present trial, increased muscle crude protein content at week 12 was observed for the AL4 and AL8 groups. The muscle protein content increased from 17% in the initial population to approximately 18% at week 8 and 12, respectively. Though the results indicate some minor differences among the dietary groups the main trend was that protein content increased slightly with growth. The reduction in muscle mineral content (ash) from the start to the end of the trial found for the algae groups reflects the lower mineral content in these diets.

Carotenoids are used in health foods, food coloring, cosmetics, vitamin supplements and feed additives (Yaakob et al., 2014). The yellow-pigmented carotenoid lutein is produced by several species of microalgae including *Scenedesmus* sp. (Chan et al., 2013). The change in skin pigmentation observed for the algae-fed wolffish could be caused by deposition of this carotenoid. High lutein was also found in rainbow trout (*Oncorhynchus mykiss*) with yellow flesh discoloration (Welker et al., 2001). Yellow discoloration of flesh will reduce the market value of rainbow trout (Skonberg et al., 1998), but it is not known how skin discoloration would affect the market value of spotted wolffish if skin is present in the product. Changed skin pigmentation with increasing algae inclusion have also been reported for similar trials with other algal species (Tulli et al., 2012, Walker and Berlinsky, 2011), but is not reported from other experiments using *Scenedesmus* sp. (Bawdy et al. 2008, Vizcaíno et al., 2014).

All dietary groups seemed to perform within the normal range for juvenile spotted wolfish, but the reduced condition factor observed for the two highest algae inclusion groups may indicate a negative effect of algae replacement. Though condition factor was reduced in fish fed algae diets, the values were higher than previously reported for juvenile spotted wolffish. Foss et al. (2001) reported condition factors of 1.08 for 206 g wolffish reared at 34‰ salinity, similar to other reports (Foss et al., 2003, Tremblay-Bourgeois et al., 2010). However, as the diets were nearly identical in composition, it can also be hypothesized that the lower CF can be explained by poorer digestibility of the microalgae diets. The cell walls of microalgae can be difficult to digest for fish, and if these are inefficiently disrupted during feed processing or in the digestive tract it can lead to reduced availability of the nutrients and energy in the diet. Reduced digestibility has been reported from similar studies with microalgae replacing fishmeal (e.g. Tibaldi et al. 2015, Tulli et al., 2012). This may also explain the observed differences in muscle proximate composition in the present experiment.

Reduced HSI was observed for all treatments, which correlates with other reports of algae-fed fish (Tulli et al., 2012, Vizcaíno et al., 2014, Walker and Berlinsky, 2011). Patterson and Gatlin (2013) reported lower HSI in fish fed non-extracted compared to those fed lipid-extracted algae. Walker and Berlinsky (2011) suggested that the lower HSI observed in their study was a result of starvation of the fish. As reduced hepatosomatic index at the end of the trial was found also for the control treatment it could indicate that the feed composition of the diets used in the present experiment was suboptimal for the fish. Presently, no tailored feed for cultured wolffish exist and the knowledge about their nutritional demands is still quite undescribed. The macronutritional profile of the diets of the present study were formulated based on earlier feeding studies with wolffish. Jonassen (2002) indicated reduced growth in juvenile wolffish fed high fat diets (20%) compared to low fat diets (15%). Papoutsoglou and Lyndon, 2006 reported no difference in growth between high (45%) and low (38%) protein inclusion in the diet. However, this trial lasted only 18 days, which may be too short to make conclusions about the performance of the fish. Protein rich diets (55-62%) are generally used for the spotted wolffish (Foss et al., 2004). Further investigation of the nutritional requirements of spotted wolffish will be necessary in future studies.

5. Conclusion

The present study investigated the effects of dietary microalgae on fast muscle cellularity in wolffish. The results indicated that diet affected fast muscle cellularity as fish fed the control and AL4 group had higher hypertrophic growth than those fed the AL8 and AL12 diets. The muscle protein and lipid increased for all the diet groups during the course of the experiment, while mineral content was reduced for the algae-fed groups. Reduced hepatosomatic index observed for all dietary groups indicate that the energy supply was suboptimal for the growing fish. A reduction in condition factor in fish fed the high algae diet indicated reduced utilization of energy. Investigations of nutrient composition as well as the capacity of wolffish to utilize microalgae is warranted in future studies.

Acknowledgements

This research was supported financially by Nordland County, Norway (91031, “Stipendiatprogram Nord Universitet” and “Mikroalger som fôringrediens), Aminor AS and Nord University. The authors would like to thank the technicians at FBA for fish rearing and technical assistance. Peter S. C. Schulze and Michael Daniel Streicher are acknowledged for advice during the statistical analysis and graphical output.

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