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Fish welfare, fast muscle cellularity, fatty acid and body-composition of juvenile spotted wolffish (*Anarhichas minor*) fed a combination of plant proteins and microalgae (*Nannochloropsis oceanica*)

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ABSTRACT

Spotted wolffish (*Anarhichas minor*) is a promising candidate for diversification of cold-water aquaculture. An increased knowledgebase is needed concerning the capacity of spotted wolffish to utilize a variety of feed ingredients such as microalgae and terrestrial plants. The aim of the study was therefore to investigate the effect of incorporating graded levels of microalgae (*Nannochloropsis oceanica*) on fish welfare indicators (growth, hepatosomatic index, hematological parameters), fast muscle cellularity, chemical and fatty acid composition. Three isonitrogenous and isocaloric diets were formulated; one control diet (0% *N. oceanica*) and two diets with low (7.5% *N. oceanica*) and high (15% *N. oceanica*) levels of microalgae replacing fishmeal and wheat in the diets. After 12 weeks of feeding, the fish showed low growth compared to previous wolffish studies (0.26% SGR). However, this effect was not treatment dependent as there were no differences in growth or fast muscle cellularity among the three treatment groups and hematological parameters showed no indications of stress in the fish fed microalgae. Hepatosomatic index decreased over the course of the experiment for all treatment groups; a significantly larger reduction was noted in the algae-fed fish compared to the control. The omega-3 fatty acid EPA increased in the whole body of the fish fed diets containing microalgae. The results suggest that spotted wolffish has potential to utilize inclusions of up to 15% of the microalgae *N. oceanica*.

1. Introduction

The continuously increasing demand for fish and shellfish for human consumption has led to exploitation and even over-exploitation of wild fish stocks (FAO, 2018). Thus, a further increase in the harvest of wild fish populations for fish-feed is not sustainable and instead, the utilization of alternative feed ingredients needs to be investigated. Ingredients such as terrestrial plants, marine invertebrates, macro- and microalgae as well as insects are examples of novel ingredients with potential as feed ingredients for cultured fish (Naylor et al., 2009). The use of plant proteins has allowed the aquaculture industry to grow without increasing the pressure on wild fisheries. However, the sustainability of land-based protein sources is debatable as these compete with land area for human food production and depend on the use of fresh water, both of which are limited resources. Also, complete replacement of fishmeal with plant protein has proven difficult for many species, particularly for marine fish (Gatlin et al., 2007; Hardy, 2010). Compared to fish-oil and fishmeal, challenges with terrestrial plant-ingredients include imbalanced amino acid composition, high levels of carbohydrates and potential presence of anti-nutritional factors that may have adverse effects on gut health, digestion and utilization of nutrients (Bakke et al., 2014; Krogdahl et al., 2010; Marjara et al., 2012). Omega-3 (n-3) fatty acids, particularly eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids, are essential nutrients for both fish and humans (Calder, 2014; Tocher, 2015). Fish, and especially fatty fish like Atlantic salmon (*Salmo salar*), are the main dietary source of these polyunsaturated fatty acids (PUFA's) for humans (Tocher, 2015). However, due to the increased use of terrestrial plant-ingredients in salmon farming, not only are terrestrial fatty acids in salmon significantly increasing but the DHA and EPA levels are simultaneously decreasing (Sprague et al., 2016). To ensure that the nutritional value of the final product consumed by humans is not compromised, future aquaculture production cannot rely on land based plant ingredients alone.

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Microalgae are unicellular photoautotrophic organisms and are primary producers of the long-chain PUFA's in the marine environment, including DHA and EPA (Adarme-Vega et al., 2012). Microalgae are cultivated for their high content of lipids and PUFAs, balanced amino acid profile, antioxidants and vitamins, which are suitable not only for animal feeds, but are also used in health food products and cosmetics (Brown et al., 1997; Skjånes et al., 2013; Yaakob et al., 2014). They can be produced in salt- and wastewater or be cultivated on land areas unsuited for other types of agriculture, making microalgae very promising as feed ingredients for future aquaculture production (Collotta et al., 2016; Marjakangas et al., 2015). The microalgae *Nannochloropsis oceanica* is a good source of PUFA, especially EPA, making it a promising feed ingredient for fish (Hulatt et al., 2017; Ma et al., 2014). It is commonly used to grow rotifers for finfish hatcheries (Hemaiswarya et al., 2011). Inclusions of defatted *N. oceanica* have been successfully tested in the diet of post-smolt Atlantic salmon at a modest inclusion level of 10% with no adverse effects on fish health (Sørensen et al., 2017). Dietary inclusion of *N. oceanica* at a level of 30% in the diet of post-smolt Atlantic salmon has also shown promising results in terms of feed digestibility (Gong et al., 2018). Gbadamosi and Lupatsch (2018) found improved protein retention efficiency and a beneficial fatty acid profile in Nile tilapia (*Oreochromis niloticus*) fed *Nannochloropsis salina* replacing both fish and soybean products.

Spotted wolffish (*Anarhichas minor*) is a bottom-dwelling marine finfish, native to the North Atlantic and Barents Sea, with promising potential for cold-water aquaculture production (Foss et al., 2004). Countries like Canada, Norway, Iceland, Sweden and even Chile have been interested in production of spotted wolffish, but Norway is currently the only country with commercial production of wolffish, at Aminor AS (Halsa, Norway) with a current yearly concession of 500 tons (Aminor, 2018). Favorable traits of the spotted wolffish include high growth-rate, husbandry-suited behavior, late sexual maturation and ability to tolerate and adapt to a wide range of certain water quality parameters, such as tolerance for salinities between 7 and 35‰, hyperoxic conditions (100.5 mg O_2 kg^{-1} h^{-1}) and hypercapnia (1.1–33.5 mg CO_2 L^{-1}) and the ability to sustain high unionized ammonia (Foss et al., 2001; Foss et al., 2003a; Foss et al., 2003b; Foss et al., 2004). The possibility to use formulated diets directly at start-feeding, reduces the costly and labor intensive use of live-feeds in the hatchery phase of many other marine species (Falk-Petersen et al., 1999). Spotted wolffish have greater potential in aquaculture compared to the closely related common wolffish (*Anarhichas lupus*) because it reaches slaughter weight of 3–4 kg after 3 years and has higher fillet-yield (Hansen and Falk-Petersen, 2001; Foss et al., 2004). As an added-value product, their skin can be tanned and used as exclusive leather that is stronger than most other leathers (RUBIN, 2001).

Spotted wolffish have been shown to tolerate replacement of up to 12% of fishmeal with the microalgae *Scenedesmus obliquus* (Knutsen et al., 2019). The results of the study showed that spotted wolffish had potential to tolerate alternative feed ingredients. However, the diets in the study of Knutsen et al. (2019) contained high amounts of fishmeal (68–80%). An unexpected deposition of carotenoids in the skin of the fish was also observed in Knutsen et al. (2019). It was therefore decided not to continue with *S. obliquus*, but instead investigate another promising source of microalgae. In the present study, we aimed to evaluate the use of the microalgae *N. oceanica* for spotted wolffish using diets with lower fishmeal inclusion (22.5–30%) in combination with plant based ingredients (about 50%) in order to better mimic the present and future conventional feeds. *N. oceanica* have high content of PUFA's and may therefore have a greater potential in feed for marine species. The objectives of the present study was to investigate inclusion of graded levels (0, 7.5 and 15%) of the microalgae *N. oceanica* in a mixed plant- and fish-based diet for juvenile spotted wolffish. The diets were evaluated through (i) overall biometric gains and somatic indexes, (ii) hematological parameters (iii) fillet and whole body proximate chemical composition, (iv) fast muscle growth dynamics, and (v) fillet, liver and whole body fatty acid profile.

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

	Treatment diet		
	CTR	N7.5	N15
Ingredients (g 100 g^{-1} diet)			
Nannochloropsis oceanica[a]	0.0	7.5	15.0
Fishmeal LT70[b]	30.0	26.3	22.5
Fish oil[c]	13.0	13.0	13.0
Wheat meal[d]	14.3	10.6	6.8
Soy protein concentrate[e]	12.0	12.0	12.0
Pea protein concentrate[f]	12.0	12.0	12.0
Potato concentrate[g]	12.0	12.0	12.0
Wheat gluten[h]	5.0	5.0	5.0
Antioxidant powder[i]	0.2	0.2	0.2
MCP[j]	0.5	0.5	0.5
Micro-ingredients[k]	1.0	1.0	1.0
Proximate composition of diets (g 100 g^{-1})			
Crude fat	15.9	16.6	17.3
Crude protein	53.3	52.9	52.5
Fiber	1.2	1.1	1.0
Starch	9.3	8.0	7.7
Ash	6.2	7.3	8.5
Total phosphorus	1.1	1.0	0.9
Water	8.4	8.8	5.4
Energy (KJ g^{-1})	21.7	21.1	21.3

Treatment diets: CTR: control. N7.5: 7.5% *N. oceanica* inclusion. N15: 15% *N. oceanica* inclusion.

[a] 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).

[b] Protein: 70%; lipid: 5.8% (Sopropeche, France).

[c] SAVINOR UTS, Portugal.

[d] Protein: 11.7%; lipid: 1.6% (Casa Lanchinha, Portugal).

[e] Soycomil-P. Protein: 63%, lipid: 0.9% (ADM, The Netherlands).

[f] NUTRALYS F85F. Protein: 78%, lipid: 1% (ROQUETTE Frères, France).

[g] Prostar. Protein: 81%, Lipid: 3.1% (AVEBE, The Netherlands).

[h] VITAL. Protein: 83.7%, Lipid: 1.4% (ROQUETTE Frères, France).

[i] Paramega PX (KEMIN EUROPE NV, Belgium).

[j] Monocalcium phosphate. Phosphorus: 22%, Calcium: 16% (Fosfitalia, Italy).

[k] Vitamin & Mineral Premixi: Vitamins (IU or mg kg-1 diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 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.

2. Materials and methods

The experiment was approved by the Animal Welfare Committee at FBA, Nord University and was conducted in 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 experiment in accordance with the principle of the 3R's (Russell et al., 1959).

2.1. Experimental diets and feeding experiment

Three isonitrogenous and isocaloric diets were formulated; one control diet (CTR, 0% *N. oceanica*) and two diets with low (N7.5, 7.5% *N. oceanica*) and high (N15, 15% *N. oceanica*) levels of microalgae

replacing fishmeal and wheat in the diets. All three diets were formulated to have 40% of the protein sourced from fishmeal/algae and with the remaining 60% of the protein being of plant origin; wheat gluten, soy protein concentrate, pea protein concentrate and potato concentrate (Table 1). The diets were extruded into 5 mm pellets by Sparos Lda. (Olhão, Portugal). Prior to use, diets were stored at 4 °C and in the dark. During the course of the experiment, diets were stored at room temperature, in air-tight and light protected containers. The current study was carried out at Nord University research station, Mørkvedbukta (Bodø, Norway). Juvenile spotted wolffish (*Anarhichas minor*) with an initial mean weight of 625 ± 7.44 g, were provided by Aminor AS (Halsa, Norway). For each treatment, fish were randomly distributed into triplicate groups in 9 circular glass fiber tanks (45 fish per tank, 1 m^3). Initial stocking density of fish was 23 kg m^{-2} and this decreased to 21 kg m^{-2} towards the end of the experiment and was hence below the upper stocking density threshold of 40–60 kg m^{-2} for this size class of spotted wolffish suggested by Jonassen (2002). Water quality parameters was also within the recommendation from the literature for this species (Foss et al., 2001; Foss et al., 2003a; Foss et al., 2004). Fish were supplied with flow through, filtered (200 μm) and aerated seawater (34‰) at stable temperature (7.49 ± 0.01 °C), oxygen (87.29 ± 0.13%) and flow 1500 l/h, from 250 m depth in Saltenfjorden. Fish were kept under continuous light and fed the experimental diets in excess through automatic feeders at a feeding rate of 1.3% body weight per day between 08:00 and 21:00 during the experiment. The feeding experiment lasted 12 weeks, from January 4th to April 4th 2017.

2.2. Sampling, growth data and biometrical data collection

Individual weight and length was recorded for all fish at week 0, 6 and 12 of the experiment to determine weight and length gain, growth rate and condition factor which was calculated following the formulas:

- Weight gain (WG, %) = ((final mean weight – initial mean weight)/initial mean weight) * 100;
- Specific growth rate (SGR, % day^{-1}) = 100 × ln[final mean weight (g)/initial mean weight (g)]/days;
- Condition factor (CF) = [fish weight (g)/total length $(cm)^3$] × 100.

At each sampling point, randomly selected fish from each tank (N = 13 per tank) were anaesthetized with MS222 (tricaine methanesulfonate, 0.14 g/l) and then sacrificed by cranial concussion. Blood samples were collected from all sacrificed fish immediately. Four of the fish per treatment were frozen whole, at −20 °C, and later homogenized using a meat grinder for analysis of whole body proximate composition. For the other nine fish, the liver was removed, weighed for determination of hepatosomatic index and frozen at −20 °C for later analyses of fatty acid profiles. Hepatosomatic index (HSI) was calculated as [liver weight (g)/fish weight (g)] × 100. From five of these fish, muscle fillets were removed, frozen and later homogenized using a meat mincer and subsequently analyzed for muscle proximate composition and fatty acid profiles. From the four remaining fish, muscle was sampled for analysis of white muscle cellularity (described in section 2.6). Feed samples were stored at −20 °C and later homogenized for analyses of proximate composition, energy, fatty acid and amino acid profiles.

2.3. Blood sampling and plasma analysis

Blood samples were obtained by puncture of the caudal vessels using heparinized syringes and immediately centrifuged (4 °C, 6000 rpm, 10 min) to obtain plasma. The plasma was subsequently snap-frozen and stored at −80 °C until further analyses.

Plasma cortisol was analyzed, in un-extracted plasma, using a radioimmunoassay described by Young (1986), modified by Sundh et al. (2011) and using cortisol antibodies (code: S020; Lot: 1014–180,182) purchased from Guildhay Ltd. (no longer in business). Plasma glucose and lactate levels were measured using commercially available enzymatic kits (Sigma-Aldrich, St Louis, USA and Instruchemie, Delfzijl, The Netherlands), with protocols adapted to a 96-well microplate reader (Schram et al., 2010). Plasma osmolality was measured using a cryoscopic osmometer Advanced Model 3320 Micro-Osmometer4 (Advanced Instruments Inc., Norwood, USA). Deionized water (0 mOsmol/kg) and a standard solution (290 mOsmol/kg) were used as reference. Plasma pH and concentrations of electrolytes (Na^+, Cl^-, K^+ and Ca^+) were determined from 100 μl plasma samples using an electrolyte analyzer based on ion selective electrode technology (Convergys® ISE comfort Electrolyte Analyzer, Convergent Technologies, Cölbe, German) (Brijs et al., 2018).

2.4. Proximate chemical composition

All analyses of proximate chemical composition of feed, fish whole body and fillets were performed in duplicates. In short, moisture was determined by drying sample (5 g) to constant weight (105 °C, 20 h) (ISO 6496:1999). Ash was determined by burning sample (5 g) to constant weight (540 °C, 16 h) (ISO 5984:2002). Crude protein was determined using the Kjeldahl titration method (N × 6.25, Kjeltec™ 2300, Foss, Sweden, Bradstreet, 1954) (ISO 5983:1987), where protein is estimated from the nitrogen in the sample (Bradstreet, 1954; Kjeldahl, 1883). Crude fat was determined using the diethyl ester extraction method, according to the Norwegian Standard Association (1994). Gross energy of feed was determined using a bomb calorimeter (IKA C200 bomb calorimeter, Staufen, Germany) (ISO 6492:1999).

2.5. Fatty acid and amino acid profile

Prior to fatty acid analysis, samples from feed, muscle, whole fish and liver were freeze dried (VirTis benchtop K Mod. 2KBTXL-75 with D2.5E Vac Pump, SP Industries, Warminster, USA) and homogenized. Of the freeze dried homogenate, N = 5 fish per tank for liver and muscle and N = 4 fish per tank for whole fish was pooled (100 mg per fish). All analysis was in addition performed in duplicates. Extraction of lipids was done according to Bligh and Dyer (1959) and hydrolysis of lipid as described by Metcalfe et al. (1966). After sample preparation all samples were frozen at −80 °C. Gas Chromatography analysis of fatty acids was automatically performed using a Scion 436-GC (Agilent Technologies, USA) and by reference to a known standard (Fame mix2, Absolute standards, Inc., USA). Fatty acids were measured by peak integration and expressed as relative area percentage on the total fatty acid area by using the software Compass CDS Bruker Co-operation chromatography data system (Scion Instruments, UK).

Amino acid profiles of the feed were analyzed at Eurofins Food and Feed Testing (Moss, Norway). In short, samples for tryptophan analysis were subjected to alkaline hydrolysis in barium hydroxide followed by separation by reversed phase C18 column HPLC. Free/bound tryptophan was determined by fluorescence detection (280/356 nm). Samples for cysteine and methionine analysis were oxidized with hydrogen peroxide and formic acid. Samples for analysis of the remaining amino acids were hydrolyzed in aqueous hydrochloric acid. Amino acids were then separated in an amino acid analyzer with an ion-exchange column. Detection of amino acids was performed employing a post column derivatization with ninhydrin reagent and measured at 440 and 570 nm.

2.6. Fast muscle cellularity

Workflow for sampling and analysis of fast muscle cellularity is illustrated in Fig. 1. Myotomal steaks (5 mm thickness) were cut prior to the posterior ventral fin and photographed for image analysis (ImageJ, NIH, USA) to measure white muscle total cross-sectional area (TCA). From the left side of each steak blocks of white muscle (n = 6,

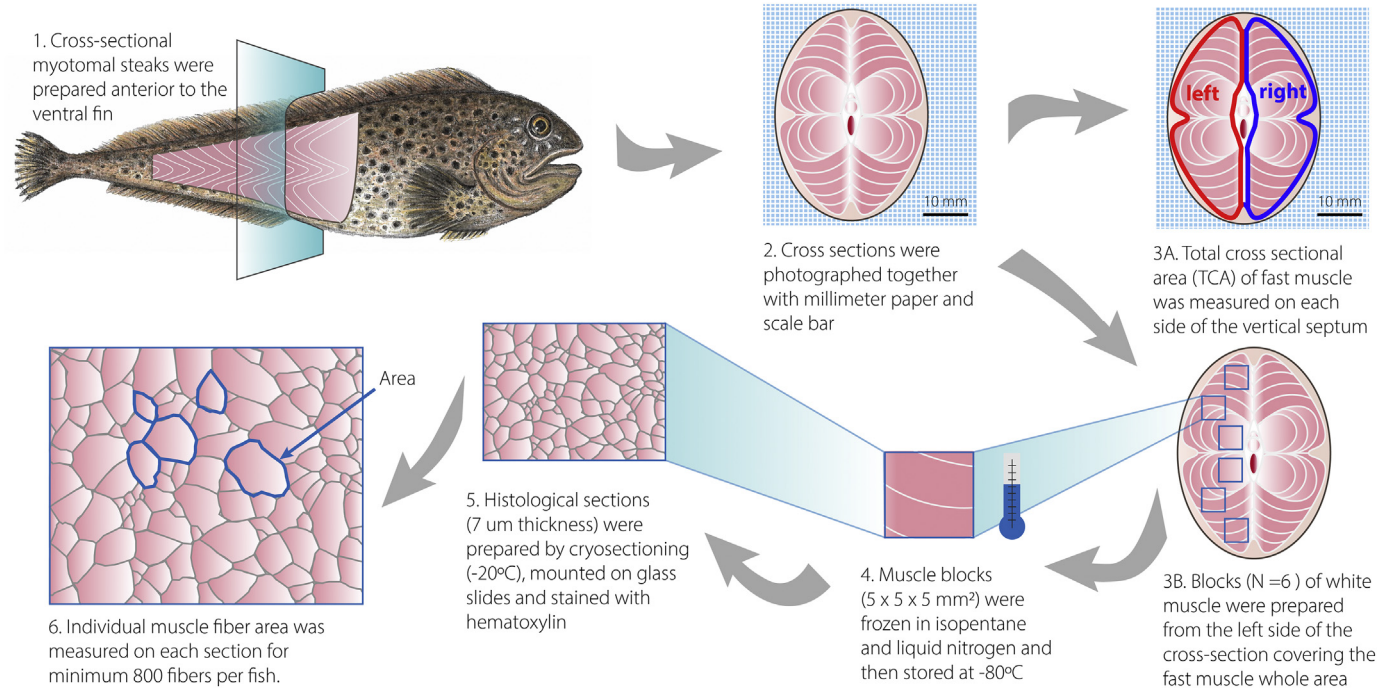

Fig. 1. Workflow for sampling and analysis of fast muscle cellularity.

5 × 5 × 5 mm) were prepared, covering the whole cross-sectional area. Muscle blocks were mounted on cork sheet (1.5 × 1.5 cm), covered in Cryomatrix (Shandon Cryomatrix, Thermo Scientific, USA) and frozen in liquid nitrogen for 45 s in 2-methyl butane (isopentane, VWR chemicals, USA) to near the freezing point (159 °C) and thereafter stored at −80 °C. Sections of muscle (7 µm) were prepared from the blocks at −20 °C using a cryostat (Cryostar NX50, Thermo Scientific, USA) and stained with Hematoxylin (Merck Chemicals, Germany). Sections were imaged using a microscope (Axioskop2, Carl Zeiss, Germany) and camera (Axiocam HRC, Carl Zeiss, Germany). Area of a minimum of 800 individual fast muscle fibers was measured using the software AxioVision 4.8 (Carl Zeiss, Germany) and individual fast muscle fiber diameter was calculated from the measured fiber area.

From the distribution of muscle fibers, recruitment of new fibers (hyperplasia) was evaluated from the presence of fibers with diameter < 20 µm. Hypertrophy was evaluated from the average diameter of the 95th percentile of the muscle fiber population. Muscle fiber number (FN) was calculated from the formula $FN = 10^6 \times$ (TCA × number of counted fibers/sum area counted fibers). Muscle fiber density (FD) was calculated from the formula $FD = 10^6 \times$ (number of counted fibers/sum area counted fibers). Daily fiber recruitment 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.

2.7. Statistical analysis

Data were tested for normality by the Shapiro-Wilk test as well as from visual inspection using density histograms and qq-plots. Homogeneity of variance was tested using Levene's test. A one-way ANOVA was used for the statistical analysis of differences between groups of the whole dataset. When the ANOVA showed significant differences, the Tukey method of multiple comparisons was used among means. A Kruskal-Wallis one-way analysis of variance on ranks was used when the data did not meet the ANOVA assumptions. When the Kruskal-Wallis test showed significance, Dunn's method of multiple comparisons was used among medians. To study the changes taking place during the course of the experiment, individual groups were compared with week 0 using a *t*-test, if the assumptions for the test were met. A Welch test was used if the data did not have homogeneity of variance and a Mann-Whitney *U* test was used if the data was not normally distributed.

Distributions of white muscle fiber diameters were analyzed using nonparametric statistical techniques fitting smoothed probability density functions (pdfs) to the measurements using a kernel function (Bowman and Azzalini, 1997), as described by Johnston et al. (1999). Fish muscle growth is highly dependent on fish size (e.g. Weatherly et al., 1988). Because of this, $N = 6$ fish of equal total length (40.14 ± 0.24 cm) were selected for analysis from each treatment group and will be the main focus of this study. Since no difference in mean lengths were found among the treatment groups, fish with lengths close to the mean length of all fish (40.02 cm) were chosen. Smoothed pdfs were fitted to the fish in each group as well as the group mean. A visual indication of potential significant differences in the white muscle fiber populations was created using bootstrap techniques for plotting approximate variability bands around the group pdfs using the mean smoothing parameter. In addition, a Kolmogorov-Smirnov two-sample test was used to test if the pdf of the treatment groups were equal over all diameters. The same analysis was also performed with the full dataset of $N = 12$ fish per treatment group. Gender effects were evaluated by splitting the dataset into males and females and analyzing the white muscle fiber diameters irrespective of treatment, as no differences were observed among treatment groups. Statistical analysis was performed using R-3.4.1 (R Core Team, 2017). Significant differences were considered when $p \leq .05$. Data are presented as means ± SEM.

3. Results

3.1. Experimental diets

All three experimental diets were isonitrogenous and isocaloric (Table 1). The diets were also balanced in terms of fiber and total phosphorous. The algae diets had higher ash and lower starch than the control diet. Water content was balanced between the control and N7.5 treatment, but was lower in the N15 treatment. Amino acid profiles of the three experimental diets (Table 2) were similar among diets, except

Table 2
Amino acids (g 100 g^{-1} diet) of the experimental diets containing different levels of microalgae (*Nannochloropsis oceanica*) as a replacement for fishmeal.

	Treatment diet		
Amino acid	CTR	N7.5	N15
Asparagine	5.15	5.07	5.24
Serine	2.49	2.44	2.53
Glutamic acid	8.60	8.36	8.46
Proline	2.99	2.99	3.26
Glycine	3.14	3.06	3.16
Alanine	2.68	2.66	2.77
Valine	2.50	2.48	2.57
Isoleucine	2.19	2.17	2.21
Leucine	4.02	3.99	4.06
Tyrosine	1.85	1.82	1.89
Phenylalanine	2.54	2.45	2.52
Histidine	1.09	1.06	1.08
Lysine	3.54	3.40	3.40
Arginine	3.21	3.13	3.21
Tryptophan	0.516	0.557	0.584
Cysteine	0.591	0.577	0.589
Methionine	0.998	1.19	1.26
Hydroxyproline	0.278	0.336	0.634
Ornitine	< 0.05	< 0.05	< 0.05
Treonine	2.15	2.12	2.19

Table 3
Fatty acids (g 100 g^{-1} diet) of the experimental diets containing different levels of microalgae (*Nannochloropsis oceanica*) as a replacement for fishmeal.

	Treatment diet		
Fatty acid	CTR	N7.5	N15
C14:0	5.25 ± 0.08	5.43 ± 0.05	5.57 ± 0.05
C16:0	19.76 ± 0.12	20.49 ± 0.25	20.91 ± 0.05
C18:0	5.05 ± 0.02	5.14 ± 0.14	5.23 ± 0.07
C16:1	6.24 ± 0.08	7.23 ± 0.04	8.42 ± 0.06
C18:1n-11	15.87 ± 0.18	15.63 ± 0.20	15.98 ± 0.00
C18:1n-9	3.49 ± 0.11	n.a	n.a
C20:1n-9	5.08 ± 0.41	5.17 ± 0.45	n.a
C22:1n-9	5.46 ± 0.07	5.52 ± 0.03	5.43 ± 0.06
C18:2n-6	8.41 ± 0.03	9.42 ± 0.10	9.87 ± 0.11
C20:5n-3 (EPA)	10.19 ± 0.01	11.18 ± 0.19	13.44 ± 0.02
C22:6n-3 (DHA)	15.18 ± 0.14	14.80 ± 0.16	15.16 ± 0.10
Σ EPA + DHA	25.37 ± 2.49	25.98 ± 1.81	28.6 ± 0.86
Σ Saturated	30.06 ± 4.87	31.06 ± 5.02	31.71 ± 5.17
Σ Monounsaturated	36.14 ± 2.21	33.55 ± 2.46	29.83 ± 3.14
Σ Polyunsaturated	33.78 ± 2.03	35.4 ± 1.58	38.47 ± 1.56
n-6/n-3	1/3	1/3	1/3

for methionine and hydroxyproline which was higher in the algae diets than the control, and highest in the N15 diet. Fatty acid composition varied slightly among diets (Table 3). The algae diets especially N15, had higher amount of PUFAs compared to the control diet, likely due to the higher content of linoleic acid (C18:2n-6) and EPA. Fatty acids were overall well balanced among the diets, but the algae diets had slightly higher content of C16:1 and C16:0.

3.2. Biometrics

Biometrical data are presented in Table 4. The three treatment groups had overall similar performance throughout the experiment. At the end of the experiment the ANOVA showed no difference in mean body weight, length, CF, WG and SGR between the treatment groups ($p > .05$). The CF and HSI was significantly lowered for all groups at week 12 compared to the start of the experiment ($p < .05$). HSI was also significantly lower in the N7.5 group compared to the control group at week 12 ($p = .01$). As no treatment effects were observed, regarding growth parameters, gender effects were analyzed using the entire data material and revealed that males had significantly higher mean weight (829.7 ± 19.1 g) compared to females (742.5 ± 20.7 g) at the end of the trial ($p = .004$).

3.3. Plasma biochemistry

Plasma biochemistry is presented in Table 5. There were no differences in plasma cortisol or glucose levels either between sampling-points or between treatment groups ($p = .12$). Plasma lactate levels was not different among groups within each sampling point, but was lower for all groups at week 12 compared to week 6 ($p < .0001$). Osmolality was not different between groups at week 6, but at week 12 it was significantly higher in the control and N15 group as compared to the N7.5 group.

3.4. Proximate composition

The proximate composition of whole fish and muscle samples is presented in Table 6. When comparing with the start of the experiment, whole body crude protein was lower for the control and N7.5 group ($p = .02$ for both) at week 6. At week 12 there were no differences in whole body crude protein among dietary groups, compared to week 0, but the N7.5 group had lower whole body crude protein compared to the control group ($p = .02$). Whole body crude lipid was unaltered both among treatment groups and over time of the experiment. Interestingly, muscle crude lipid increased for all groups at the end of the experiment although no difference among treatments was observed. Whole body ash was also lower in the N7.5 group compared to the N15 group at week 12 ($p = .004$). No differences were observed in muscle ash or moisture nor in whole body moisture.

3.5. Fast muscle cellularity

Fast muscle cellularity is presented in Table 7. There was no difference in TCA among treatments at week 12, but both algae-groups had significantly higher TCA at termination compared to the start of the experiment ($p = .02$ for both). No differences in fiber number among treatment groups were observed, but the control group had lower fiber number at week 12, compared to week 0 ($p = .048$). Although not significant, the N7.5 group also had a slightly lower fiber number compared to week 0 while those fed N15 had higher fiber number compared to week 0. Fiber densities were similar among groups at week 12, but all groups had significantly lower fiber density at week 12 compared to week 0 ($p = .01$, $p = .01$ and $p = .04$ for the control, N7.5 and N15 group, respectively). Mean diameter of the 95th percentile, 90th percentile and 75th percentile had increased for all feeding groups at week 12 ($p < .05$), but mean diameter increased only for the N7.5 and N15 groups ($p = .01$ and $p = .04$ respectively). All feeding groups showed decreased proportion of fibers with D ≤ 20 μm and increased proportion of fibers with D > 200 μm at the end of the experiment ($p < .05$). The probability density function (Fig. 2) showed no difference in muscle fiber distributions among the feeding groups, both when selecting $N = 6$ fish of equal total length (40.14 ± 0.24 cm) from each treatment group and when the full dataset ($N = 12$ fish per treatment group) was analyzed (data not shown). The probability density function of males and females (irrespective of treatment group) revealed no sex differences in muscle growth (data not shown).

3.6. Fatty acid composition of whole body, liver and muscle

Fatty acid composition of muscle, liver and whole body are presented in Table 8. The fatty acid profile differed slightly among the different tissues. Fatty acid composition of whole fish was dominated by monounsaturated fatty acids, of which C18:1n-9 accounted for 73–74%. The sum of saturated fatty acids was slightly reduced from 18% to approximately 16%; sum PUFA's increased, while EPA + DHA were in

Table 4
Survival, sex, 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 (*Nannochloropsis oceanica*). Weight, length, WG, DGI and CF for week 0 and week 12 are based on measurements of all fish. All other values are based on sampled fish. Sampled fish are excluded from the calculation of survival. Values are means ± SEM. Means in the same column at the same time point with different subscript letter differs significantly ($p < .05$). Means in the same column with subscript * differ significantly from the start of the experiment ($p < .05$).

Time	Diet	Survival [%]	Sex [♂/♀]	Body weight [g]	Body length [cm]	CF	HSI	WG [%]	SGR [% day^{-1}]
Week 0	Start	n.a	19♂/17♀	624.79 ± 7.45	36.08 ± 0.13	1.31 ± 0.01	4.29 ± 0.08	n.a	n.a
Week 6	CTR	100	13♂/14♀	758.36 ± 15.99*	38.74 ± 0.25*	1.29 ± 0.02*	4.02 ± 0.07^{a}*	21.57 ± 2.13^{a}	0.41 ± 0.04^{a}
	N7.5	100	17♂/10♀	750.74 ± 15.84*	38.73 ± 0.23*	1.27 ± 0.01*	3.63 ± 0.09^{b}*	19.03 ± 1.09^{a}	0.37 ± 0.02^{a}
	N15	100	15♂/12♀	750.24 ± 17.23*	38.60 ± 0.27*	1.28 ± 0.01*	3.66 ± 0.07^{b}*	20.51 ± 0.54^{a}	0.40 ± 0.01^{a}
Week 12	CTR	96.6	15♂/12♀	785.21 ± 20.72*	39.98 ± 0.35*	1.22 ± 0.02^{a}*	3.60 ± 0.07^{a}*	26.09 ± 5.32^{a}	0.25 ± 0.07^{a}
	N7.5	100	19♂/8♀	804.69 ± 21.48*	40.18 ± 0.30*	1.22 ± 0.01^{a}*	3.32 ± 0.06^{b}*	27.42 ± 3.10^{a}	0.27 ± 0.03^{a}
	N15	94.9	15♂/12♀	791.34 ± 22.51*	39.91 ± 0.35*	1.22 ± 0.01^{a}*	3.39 ± 0.07^{ab}*	26.69 ± 7.72^{a}	0.26 ± 0.05^{a}

Table 5
Plasma biochemistry of spotted wolffish fed diets with different level of inclusion of microalgae (*Nannochloropsis oceanica*). Values are means ± SEM. Means in the same column at the same time point with different subscript letter differ significantly ($p < .05$).

	Week 6			Week 12		
	CTR	N7.5	N15	CTR	N7.5	N15
Cortisol (mmol/l)	12.62 ± 2.35	8.39 ± 1.68	12.57 ± 2.04	16.78 ± 2.68	13.80 ± 2.92	12.58 ± 2.13
Glucose (mmol/l)	0.65 ± 0.02	0.64 ± 0.02	0.62 ± 0.02	0.59 ± 0.02	0.65 ± 0.03	0.63 ± 0.03
Lactate (mmol/l)	0.71 ± 0.05	0.86 ± 0.07	0.68 ± 0.05	0.41 ± 0.05	0.43 ± 0.03	0.47 ± 0.04
Osmolality (mOsmol/kg^{-1})	353.22 ± 1.47	354.22 ± 1.53	353.43 ± 1.95	369.95 ± 2.16^{a}	358.51 ± 1.73^{ab}	362.92 ± 1.63^{b}
Cl^- (mmol/l)	134.98 ± 1.36	136.47 ± 2.41	135.66 ± 1.62	139.63 ± 0.92	139.28 ± 0.79	137.88 ± 0.83
Na^+ (mmol/l)	182.37 ± 2.78	183.74 ± 2.09	180.02 ± 2.21	190.33 ± 1.15	191.49 ± 1.26	189.06 ± 0.95
K^+ (mmol/l)	4.10 ± 0.08	4.25 ± 0.10	4.12 ± 0.06	3.83 ± 0.07^{a}	4.06 ± 0.06^{b}	3.92 ± 0.05^{ab}
Ca^{2+} (mmol/l)	1.00 ± 0.02^{a}	0.97 ± 0.03^{ab}	0.91 ± 0.01^{b}	1.05 ± 0.01^{a}	1.01 ± 0.01^{b}	0.97 ± 0.01^{b}
pH	7.29 ± 0.02	7.26 ± 0.02	7.21 ± 0.09	7.29 ± 0.01^{a}	7.30 ± 0.00^{ab}	7.32 ± 0.01^{b}

Table 6
Muscle and whole fish proximate composition [%] of spotted wolffish fed diets with different level of inclusion of microalgae (*Nannochloropsis oceanica*). Values are means ± SEM. Means in the same column at the same time point with different subscript letter differ significantly ($p < .05$). Means in the same column with subscript * differ significantly from the start of the experiment ($p < .05$).

		Crude protein		Crude lipid		Ash		Moisture	
Time	Diet	*Whole fish*	*Muscle*	*Whole fish*	*Muscle*	*Whole fish*	*Muscle*	*Whole fish*	*Muscle*
Week 0	Start	14.33 ± 0.06	16.80 ± 0.10	9.66 ± 0.25	8.36 ± 0.28	1.38 ± 0.02	1.20 ± 0.01	73.94 ± 0.21	73.16 ± 0.22
Week 6	CTR	14.05 ± 0.11*	16.33 ± 0.12*	9.89 ± 0.12	9.29 ± 0.28*	1.34 ± 0.03	1.19 ± 0.03	73.92 ± 0.18	72.85 ± 0.21
	LOW	14.06 ± 0.10*	16.34 ± 0.12*	9.83 ± 0.16	9.43 ± 0.34*	1.35 ± 0.04	1.22 ± 0.02	74.11 ± 0.23	72.79 ± 0.29
	HIGH	14.24 ± 0.10	16.11 ± 0.12*	9.89 ± 0.29	10.07 ± 0.21*	1.36 ± 0.03	1.26 ± 0.03*	73.65 ± 0.35	72.34 ± 0.19*
Week 12	CTR	14.62 ± 0.16^{a}	16.52 ± 0.14	9.73 ± 0.24	9.48 ± 0.40*	1.36 ± 0.03^{ab}	1.32 ± 0.04*	73.61 ± 0.37	72.55 ± 0.32
	LOW	14.12 ± 0.11^{b}	16.28 ± 0.10*	9.47 ± 0.26	9.58 ± 0.24*	1.27 ± 0.04^{a}*	1.24 ± 0.03	74.53 ± 0.25	72.90 ± 0.20
	HIGH	14.43 ± 0.10^{ab}	16.33 ± 0.12*	9.80 ± 0.21	9.62 ± 0.34*	1.42 ± 0.03^{b}	1.34 ± 0.05	73.94 ± 0.29	72.33 ± 0.34*

the same range during the course of the experiment. Compared to whole body, muscle fillet contained less monounsaturated fatty acids and they were less dominated by C18:1n-9. Moreover, the sum of PUFAs was 50–60% higher, and sum of EPA and DHA were in the range 43–59% higher in fillet compared to the whole body. Fatty acid profile of the liver was more similar to the fillet, but with lower content of the sum of PUFAs as well as the sum of EPA and DHA. An increased content of C18:2n-6 was observed in muscle ($p = .02$, $p = .01$ and $p = .01$ for CTR, N.7.5 and N15, respectively), liver ($p = .01$, $p = .0003$ and $p = .001$ for CTR, N.7.5 and N15, respectively) and whole fish ($p = .02$, $p = .0005$ and $p = .003$ for CTR, N.7.5 and N15, respectively) during the course of the experiment.

At termination of the experiment, there were only marginal differences in muscle fatty acid composition among the different feeding groups. The saturated fatty acid C14:0 decreased over time in both the algae groups ($p = .02$ and $p = .04$ for N7.5 and N15, respectively) and C16:0 also decreased with time in N7.5 ($p = .045$). The monounsaturated fatty acid C22:1n-9 also decreased in the muscle of the algae groups with time ($p = .03$ and $p = .01$ for N7.5 and N15, respectively).

In the liver, sum of saturated fat was reduced in all treatment groups at termination of the experiment. Liver content of the saturated fatty acid C14:0 was reduced for all groups at week 12 ($p = .004$, $p = .001$ and $p = .005$ for CTR, N.7.5 and N15, respectively). The saturated fatty acid C16:0 was reduced in liver for all groups at week 12 ($p = .002$, $p = .0004$ and $p = .01$ for CTR, N.7.5 and N15, respectively) and was also lower in N7.5 compared to N15 at week 12 ($p = .02$). The saturated fatty acid C19:0 was higher in liver of the algae-fed fish compared

Table 7
Fast muscle cellularity of spotted wolffish fed diets with different levels of inclusion of microalgae (*Nannochloropsis oceanica*). Values are means ± SEM. Means in the same row with different superscript letters differ significantly ($p < .05$). 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. Means in the same row at the same time point with different subscript letter differs significantly ($p < .05$). Means in the same column with subscript * differ significantly from the start of the experiment ($p < .05$).

Time	Week 0	Week 12		
	Start	CTR	LOW	HIGH
TCA	2041.0 ± 76.5	2271.1 ± 55.49	2369.5 ± 89.03*	2347.4 ± 94.52*
Fiber number	158703 ± 12826	151173 ± 6261*	154089 ± 9118	163561 ± 7307
Fiber density [fibers mm^{-2}]	84.1 ± 4.12	67.1 ± 3.45*	65.4 ± 3.97*	71.0 ± 4.68*
95th percentile	198.2 ± 5.46	222.1 ± 4.64*	228.5 ± 6.27*	221.5 ± 6.65*
D mean of upper 95th percentile	179.7 ± 4.76	246.0 ± 5.02*	257.2 ± 8.19*	247.9 ± 7.18*
90th percentile	121.3 ± 7.93	200.4 ± 4.64*	203.9 ± 5.33*	199.0 ± 6.08*
D mean of upper 90th percentile	204.0 ± 5.58	228.1 ± 4.80*	235.4 ± 6.57*	228.2 ± 6.68*
75th percentile	150.2 ± 4.08	167.5 ± 4.91*	168.3 ± 5.06*	161.2 ± 4.79
D mean of upper 75th percentile	179.9 ± 4.79	200.7 ± 4.57*	204.7 ± 5.50*	197.8 ± 5.81*
D max	277.4 ± 8.45	305.9 ± 6.57	329.7 ± 13.20*	313.5 ± 12.49*
Proportion [%] of white muscle fibers with				
D ≤ 20 μm	0.5 ± 0.18	1.4 ± 0.21*	1.5 ± 0.26*	1.4 ± 0.33*
D > 200 μm	5.7 ± 1.29	11.4 ± 1.78*	12.1 ± 1.72*	10.2 ± 1.42*

to the control at week 12 ($p = .039$ and $p = .046$ for N7.5 and N15, respectively). The monounsaturated fatty acid C16:1 was higher in the control and N7.5 groups at week 12 compared to the start of the experiment ($p = .04$ for both treatments). The sum of monounsaturated fatty acids in the liver were only lower at the terminal sampling compared to the start of the experiment, in the N7.5 group ($p = .049$). The monounsaturated fatty acids C20:1n-9 and C22:1n-9 were reduced in the liver of all groups during the course of the experiment ($p < .05$). In the N7.5 group, the sum of PUFA's ($p = .03$) as well as the fatty acid C22:5n-3 ($p = .04$) had increased in the liver at week 12 compared to the start of the experiment.

Whole body C14:0, C16:0 and C18:0 was reduced for all groups at termination of the experiment compared to week number 0 ($p < .05$). Although no difference was observed among groups, whole body C16:1 was reduced in both algae groups at week 12 compared to week 0 ($p = .023$ and $p = .049$ for N7.5 and N15, respectively). Whole body C18:1n-7 was also higher in N7.5 compared to the control group ($p = .02$) and C22:1n-9 increased with time for both algae groups ($p = .001$ and $p = .02$ for N7.5 and N15, respectively). The PUFA 22:5n-3 increased over time in whole body samples for all treatment groups ($p = .04$, $p = .01$ and $p = .03$ for CTR, N7.5 and N15, respectively) and whole body 20:5n-3 was increased in the algae groups at the

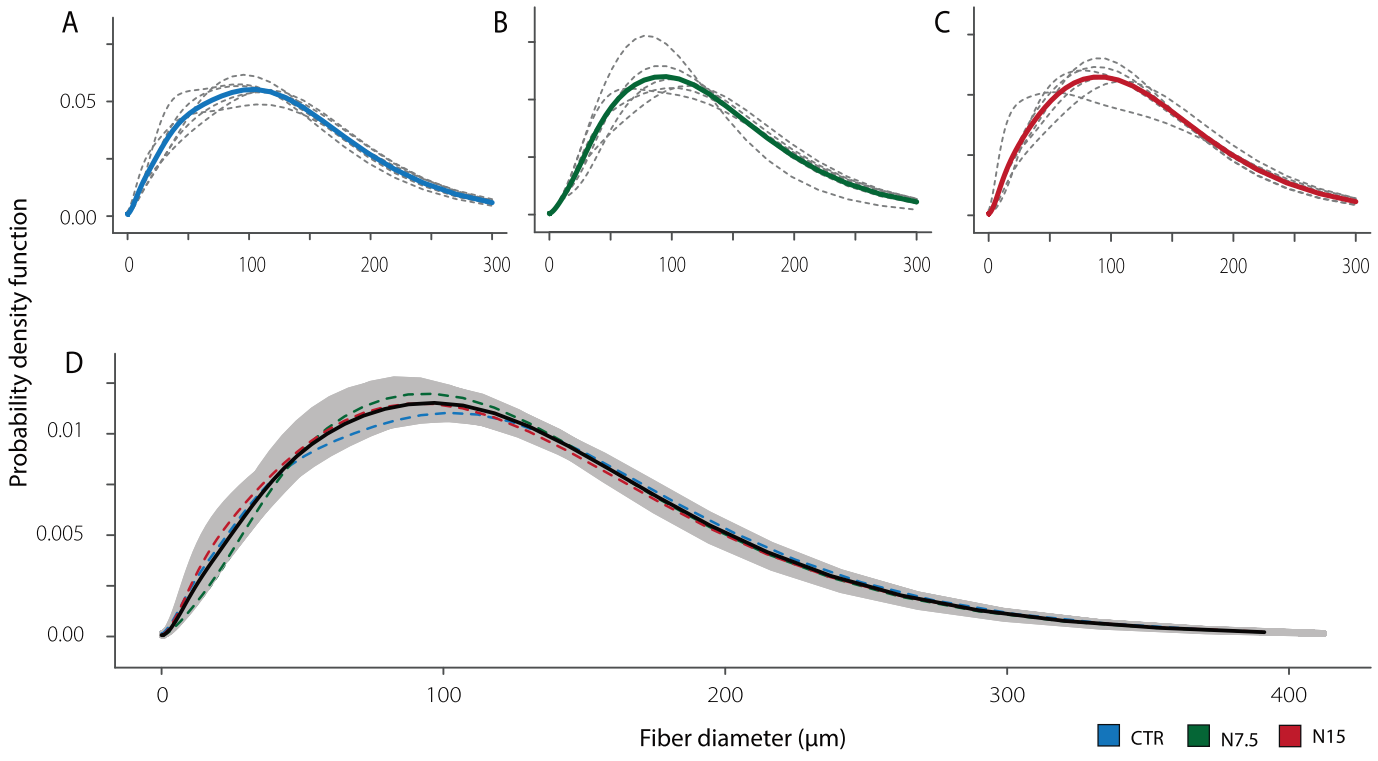

Fig. 2. Probability density functions (pdf) of fast muscle fiber diameters for spotted wolffish fed diets with different levels of the microalgae *Nannochloropsis oceanica* included in the diet. (A)-(C) shows the mean pdf for each group (solid line) as well as pdf's for individual fish in each group (dashed line). (A) Control, no algae. (B) 7.5% microalgae (N7.5). (C) 15% microalgae (N15). (D) shows the mean pdf for all groups (solid black line), mean pdf for each group (dashed lines) and approximate variability bands produced with bootstrapping techniques.

Table 8
Changes in fatty acid content (g $100\,g^{-1}$) in muscle fillet, liver and whole body of spotted wolffish fed diets with different levels of inclusion of microalgae (*Nannochloropsis oceanica*). Values are means ± SEM. Means in the same column at the same time point with different subscript letter differ significantly ($p < .05$). Means in the same column with subscript * differs significantly from the start of the experiment ($p < .05$).

Time	Week 0	Week 12		
	Start	CTR	Low	High
Muscle fillet				
C14:0	4.82 ± 0.08	4.64 ± 0.14	4.51 ± 0.03*	4.51 ± 0.07*
C16:0	14.06 ± 0.14	14.10 ± 0.25	13.57 ± 0.09*	13.84 ± 0.11
C18:0	2.39 ± 0.04	2.49 ± 0.06	2.40 ± 0.06	2.48 ± 0.06
C19:0	2.28 ± 0.14	2.15 ± 0.07	2.17 ± 0.02	2.25 ± 0.04
C20:0	2.82 ± 0.00	n.a	n.a	n.a
C16:1	8.64 ± 0.14	8.56 ± 0.19	8.40 ± 0.11	8.62 ± 0.12
C18:1n-9 + 11	26.03 ± 0.46	27.28 ± 0.45	26.70 ± 0.29	27.01 ± 0.32
C20:1n-9	4.83 ± 0.19	4.20 ± 0.33	4.48 ± 0.05	4.40 ± 0.09
C22:1n-9	4.33 ± 0.08	4.13 ± 0.33	3.97 ± 0.10*	3.88 ± 0.09*
C18:2n-6	7.01 ± 0.14	7.82 ± 0.19*	7.93 ± 0.14*	7.92 ± 0.12*
C20:5n-3 (EPA)	10.09 ± 0.17	9.82 ± 0.23	10.04 ± 0.01	10.06 ± 0.10
C22:5n-3	1.97 ± 0.07	2.86 ± 0.82	1.96 ± 0.05	1.91 ± 0.03
C22:6n-3 (DHA)	13.91 ± 0.13	14.12 ± 0.25	14.19 ± 0.04	14.10 ± 0.11
Σ Saturated	26.38 ± 1.19	23.38 ± 1.56	22.66 ± 1.40	23.07 ± 1.43
Σ Monounsaturated	44.04 ± 0.62	44.16 ± 2.01	43.55 ± 1.94	43.91 ± 1.99
Σ Polyunsaturated	32.98 ± 1.10	34.61 ± 1.24*	34.13 ± 1.33*	33.98 ± 1.30*
Σ EPA + DHA	24.00 ± 0.73	23.94 ± 0.97	24.23 ± 0.93	24.16 ± 0.91
n-6/n-3	1/4	2/7	1/3	1/3
Liver				
C14:0	4.49 ± 0.05	4.10 ± 0.06*	4.01 ± 0.03*	4.13 ± 0.06*
C16:0	14.29 ± 0.09	13.49 ± 0.10^{ab}*	13.16 ± 0.10^{a}*	13.70 ± 0.11^{b}*
C18:0	2.92 ± 0.03	2.93 ± 0.03	2.92 ± 0.04	2.96 ± 0.02
C19:0	1.98 ± 0.07	1.92 ± 0.01^{a}	2.03 ± 0.03^{b}	2.02 ± 0.03^{b}
C16:1	8.58 ± 0.11	8.15 ± 0.12*	8.16 ± 0.10*	8.47 ± 0.06
C18:1n-9 + 11	30.36 ± 0.33	31.21 ± 0.26	30.65 ± 0.59	30.21 ± 0.45
C20:1n-9	4.66 ± 0.06	4.34 ± 0.04*	4.08 ± 0.11*	4.22 ± 0.01*
C22:1n-9	4.25 ± 0.07	3.68 ± 0.09*	3.46 ± 0.08*	3.59 ± 0.04*
C18:2n-6	6.54 ± 0.08	7.11 ± 0.09^{a}*	7.57 ± 0.08^{b}*	7.42 ± 0.07^{ab}*
C20:5n-3 (EPA)	9.19 ± 0.13	9.17 ± 0.07	9.32 ± 0.01	9.38 ± 0.08
C22:5n-3	1.70 ± 0.05	1.79 ± 0.04	1.86 ± 0.02*	1.86 ± 0.06
C22:6n-3 (DHA)	12.10 ± 0.25	12.40 ± 0.10	12.80 ± 0.17	12.33 ± 0.28
Σ Saturated	23.68 ± 1.70	22.43 ± 1.39^{ab}*	22.11 ± 1.35^{a}*	22.82 ± 1.41^{b}*
Σ Monounsaturated	47.84 ± 1.96	47.38 ± 2.39	46.34 ± 2.36*	46.50 ± 2.31
Σ Polyunsaturated	29.55 ± 0.94	30.47 ± 1.16	31.54 ± 1.19*	31.00 ± 1.15
Σ EPA + DHA	21.30 ± 0.57	21.57 ± 0.73	22.11 ± 0.78	21.71 ± 0.67
n-6/n-3	2/7	1/3	1/3	1/3
Whole fish				
C14:0	1.82 ± 0.04	1.54 ± 0.05*	1.46 ± 0.00*	1.55 ± 0.01*
C16:0	11.30 ± 0.29	9.98 ± 0.27*	9.32 ± 0.03*	9.42 ± 0.09*
C18:0	4.57 ± 0.13	3.94 ± 0.16*	3.90 ± 0.04*	4.00 ± 0.04*
C19:0	n.a	0.90 ± 0.01^{a}	0.95 ± 0.02^{a}	0.98 ± 0.07^{a}
C16:1	7.20 ± 0.26	6.50 ± 0.38	6.08 ± 0.02*	6.38 ± 0.04*
C18:1n-9 + 11	45.99 ± 0.34	45.10 ± 1.86	45.60 ± 0.59	46.03 ± 1.31
C18:1n-7	4.91 ± 0.21	5.23 ± 0.04^{a}	5.56 ± 0.09^{b}	5.39 ± 0.04^{ab}
C20:1n-9	2.73 ± 0.09	2.66 ± 0.16	2.77 ± 0.01	2.72 ± 0.08
C22:1n-9	1.67 ± 0.02	1.41 ± 0.18	1.41 ± 0.03*	1.44 ± 0.08*
C18:2n-6	3.27 ± 0.12	4.02 ± 0.19*	4.51 ± 0.06*	4.46 ± 0.19*
C20:3n-3	n.a	0.87 ± 0.12^{a}	0.87 ± 0.03^{a}	1.04 ± 0.00^{a}
C20:5n-3 (EPA)	7.34 ± 0.15	7.86 ± 0.34	8.06 ± 0.10*	8.13 ± 0.29*
C22:5n-3	1.40 ± 0.07	1.71 ± 0.08*	1.80 ± 0.05*	1.76 ± 0.09*
C22:6n-3 (DHA)	7.80 ± 0.17	8.84 ± 0.39	8.01 ± 0.28	7.88 ± 0.35
Σ Saturated	17.69 ± 1.21	16.35 ± 1.08	15.63 ± 1.00*	15.95 ± 1.06*
Σ Monounsaturated	62.50 ± 3.87	60.90 ± 4.44	61.41 ± 4.48	61.95 ± 4.53
Σ Polyunsaturated	19.82 ± 0.70	23.31 ± 0.88*	23.24 ± 0.81*	23.27 ± 0.81*
Σ EPA + DHA	15.14 ± 0.14	16.71 ± 0.38	16.07 ± 0.13	16.01 ± 0.21
n-6/n-3				

end of the experiment ($p = .015$ and $p = .046$ for N7.5 and N15, respectively).

4. Discussion

In the present experiment, inclusion of the microalgae *N. oceanica* at different inclusion rates did not affect the overall performance of the wolffish. Total weight increase was on average 26% (625–794 g) during the course of the experiment with an average SGR of 0.26%. This is slightly lower than the growth rates of wolffish of similar size reported by Jonassen (2002), who found SGR values of 0.41–0.47% in wolffish growing from 450 to 850 g over 126 days. The SGR is dependent on size and developmental stage of the fish. Thus, within a species, the growth rates are expected to decrease with size (Ricker, 1979). For smaller wolffish, ranging from 60 to 140 g, SGR values of 0.8–1.2% has been reported (Foss et al., 2001; Foss and Imsland, 2002; Foss et al., 2003a).

Knutsen et al. (2019) reported an average SGR of 0.66% in wolfish growing from 140 to 250 g. The inverse relationship between size and SGR for spotted wolffish is apparent across experiments, and is in line with Imsland et al. (2006). Dupont Cyr et al. (2018) reported a fluctuating SGR for both spotted and Atlantic wolffish (weight ranging between 1 and 3 kg) associated with reproductive events. The latter study reported SGR values as low as 0.1 at certain times of the year. Males were also found to have larger weight than females (Dupont Cyr et al., 2018), in line with the results of the present study. Even though the SGR of the present study was lower than expected, similar and lower SGRs have been reported and the discrepancies may be explained by different rearing systems and conditions among the experiments.

The plasma levels of cortisol in the experimental fish were within the range considered normal for spotted wolffish of this size (~15–30 nmol/l) (Lays et al., 2009; Le François et al., 2013). In this species, stress have been reported to elicit lower plasma cortisol responses than shown in most other teleosts. Acute and severe disturbances such as air exposure or hypoxia, elicit increases of up to 80–95 nmol/l in the spotted wolffish (Lays et al., 2009; Le François et al., 2013), whereas in other teleosts, rapid responses above 276 nmol/l are generally considered to reflect an acute and severe stress response and increases up to 166 nmol/l are referred to as a mild stress response (Wendelaar Bonga, 1997). Similarly, basal plasma glucose levels in spotted wolffish are relatively low (0.3–0. 4 mmol/l (Lays et al., 2009) and comparable to species with a sedentary lifestyle and low locomotor activity (< 1 mmol/l) (Vijayan, 1994). The stress response is known to differ considerably between teleost species, depending on the ecotype and lifestyle. Benthic, sedentary species with a low metabolic rate, such as the spotted wolffish, tend to have a lower response than pelagic and active species such as salmonids (Barton and Iwama, 1991; Vijayan, 1994). The low levels of plasma cortisol and glucose observed in our study and the absence of differences between treatments both suggest that the partial substitution of a fish-based diet with microalgae did not impose stress.

There were no major differences among the three treatment groups at weeks 6 and 12 regarding plasma pH, osmolality and ion concentrations of Na^+, K^+, Cl^- and Ca^{2+}. These parameters are within the range found for this species (Na^+: 127–186 mM, Imsland et al., 2009; Magnussen et al., 2008; Tremblay-Bourgeois et al., 2010; Cl^-: 140–164 mM, Foss et al., 2001; Magnussen et al., 2008; K^+: 2,60–4,56 mM, Imsland et al., 2009; Tremblay-Bourgeois et al., 2010, osmolality: 320–356 mOsmolkg^{-1}, Foss et al., 2001; Magnussen et al., 2008, and pH: 7,12-7,42, Imsland et al., 2009). Plasma concentrations of Ca^{2+} have, to our knowledge, not been reported previously for any wolffish species, but seem to be relatively low compared to other marine species (Abbink et al., 2004; Person-le Ruyet et al., 2003; Sala-Rabanal et al., 2003). The differences in plasma lactate concentration between weeks 6 and 12 can be explained by the differences in osmoregulatory capacity seen between the two sampling points, as lactate is used as an important metabolic fuel of the chloride cells, involved in osmoregulation (Perry and Walsh, 1989). To our knowledge, it is the first time plasma concentrations of lactate are measured in spotted wolffish. As for glucose and cortisol, the values obtained for lactate are once again very low in comparison with active pelagic fish, such as the yellowtail kingfish, *Seriola lalandi* (Blanco Garcia et al., 2015), and more comparable to resting levels of benthic fish, such as turbot *Scophthalmus maximus* (Pichavant et al., 2002), which is consistent with the relatively quiet lifestyle of spotted wolffish.

The reduction in CF during the course of the experiment coincided with a reduction in HSI. Knutsen et al. (2019) also reported a reduction in HSI over time when feeding spotted wolffish another microalgae, *Scenedesmus obliquus*. The reduction in HSI could be explained by modulated lipid metabolism and increased need for lipid to be deposited in the muscle associated with growth of the fish. There were no changes in whole body crude lipid with the growth of the fish, while muscle crude lipid increased for all groups. These findings strongly suggest that lipids stored in the liver were mobilized and deposited in the muscle. The increased muscle lipid content can only be explained by the size of the fish because there were no difference among treatment groups at week 12 of the experiment. Spotted wolffish of 250 g have about 5% muscle crude lipid (Knutsen et al., 2019), while the 794 g fish in the present experiment had 9.6% muscle crude lipid; it is likely that spotted wolffish incorporate more lipid in the muscle as they increase body mass. The reduction in HSI may be explained by the increasing incorporation of microalgae in the two experimental diets. Reduced HSI has been found for Atlantic cod fed high inclusions of microalgae (30%) in the diet and for European seabass (*Sparus aurata*) fed the microalgae *Tetraselmis suecica* replacing 20% of the dietary fishmeal (Tulli et al., 2012; Walker and Berlinsky, 2011). Tulli et al. (2012) suggested that this could be related to the lowered apparent digestibility coefficient of the microalgae diet but also that it could be related to microalgae compounds with possible modulating effects on lipid metabolism. Other studies (e.g. Walker and Berlinsky, 2011) have suggested that a reduction in HSI was caused by depletion of stored hepatic lipids as a response to starvation. However, Walker and Berlinsky (2011) observed difference in HSI of 4.18 between the control treatment (HSI = 6.99) and highest algae treatment (HSI = 2.18). This was far greater than the observed difference of 0.21 between the control and highest algae treatment in the present experiment. It is also far greater than the observed difference of 0.85 between week 0 and the mean HSI of all groups at week 12 in the present experiment. Other experiments replacing fishmeal with microalgae observed no difference in HSI for red drum (Patterson and Gatlin, 2013) and Atlantic salmon (Sørensen et al., 2017). High protein feeds (> 50%) have generally been used for spotted wolffish (Foss et al., 2004), and the present diets were formulated according to this.

All three diets were isoenergetic and isonitrogeneous and had nearly identical amino acid profiles. Despite this, muscle crude protein was reduced in both the algae-fed groups and whole body crude protein was also lower in the N7.5 group, at week 12, compared to the control. This could indicate a lower capacity of the fish to absorb and utilize microalgae-protein due to the complex carbohydrates in the cell-walls of microalgae that may be hard to digest for carnivorous fish (Domozych et al., 2012; Krogdahl et al., 2005; Teuling et al., 2017; Teuling et al., 2019).

The increased content of linoleic acid (C18:2n-6) during the course of the experiment in all dietary treatments and in both muscle, liver and whole body is most likely reflecting a general increase in lipid deposition. It is well known that replacement of fishmeal and fish oil with plant based ingredients is generally reflected in the composition of fish tissue lipids, resulting in reduced levels of ω-3 (PUFAs) and increased levels of linoleic acid (C18:2n-6) (Watanabe, 1982). Overall, there were few changes in the fatty acid composition among fish fed the different experimental diets, and the two nutritionally important fatty acids EPA and DHA seemed to be conserved in all tissues during the course of the experiment. The increased retention of EPA in the whole body samples of fish fed the microalgae diets can be explained by the high EPA content of *N. oceanica*.(Hulatt et al., 2017; Ma et al., 2014). Improved retention of essential fatty acids are previously reported in studies with Atlantic salmon fed diets where fish oil was replaced by *Schizochytrium* sp. (Kousoulaki et al., 2015; Kousoulaki et al., 2016). The retention of PUFAs in fish tissues is important both for the health of the fish and for the nutritional quality of fish in the human diet (Bou et al., 2017; Mozaffarian and Rimm, 2006). *N. oceanica* has also high levels of saturated and monounsaturated fatty acids (Draaisma et al., 2013; Hulatt et al., 2017). This was not reflected in either muscle, whole body or liver samples, where the level of total saturated fatty and monounsaturated acids was either unchanged or decreased in all groups and tissues.

Replacing fishmeal with microalgae did not have any negative effects on fast muscle cellularity for spotted wolffish. Overall, the number of fast muscle fibers did not change during the course of the

experiment, except for the control group where the numbers decreased slightly with time. This was surprising as the growth pattern of juvenile fish is expected to be dominated by mosaic hyperplasia in addition to hypertrophy, as shown for several other teleost species (Stickland, 1983; Weatherly et al., 1988; Zimmerman and Lowery, 1999). In general, teleost fish cease recruiting new fibers at a size of about 44% of the final body length (Weatherly et al., 1988). Studies have also recorded a cessation of hyperplasia at a much earlier point than 44% of final length in Antarctic living species (Johnston et al., 2003). Therefore, it is not unlikely that the recruitment of new fibers in the present experiment could cease at mean length 40 cm, 22% of the spotted wolffish's maximal adult length of 180 cm (Bakketeig et al., 2017). However, despite the unexpected low fiber number, fibers < 20 μm increased in all groups at week 12, indicating a small contribution of hyperplastic growth.

Within strains of the same species, a large variation can exist in muscle fiber number and density (Johnston et al., 2000). In Atlantic halibut (*Hippoglossus hippoglossus*muscle fiber recruitment have been known to cease during periods of poor growth (Hagen et al., 2006). In the present study, the relatively low weight gain may partially explain the lack of apparent mosaic hyperplasia. In addition, halibut has the ability to grow larger fast fibers than other more active pelagic species, suggesting that fiber development is linked to the activity level, fish swimming behavior and diffusion constraints (Johnston et al., 2004; Johnston et al., 2005). Unusually large muscle fibers have also been documented for another benthic fish species, common Dab (*Limanda limanda*; Hurling et al., 1996) as well as for the Notothenioid fishes (fibers > 700 μm) which is a group of fishes living in the cold waters of the sub-Antarctic (Johnston et al., 2003). Spotted wolffish had on average a maximum fiber diameter of 317 μm at the end of the experiment, but for individual fish fiber diameters over 430 μm were observed. The findings of this study suggests that, independent of diets, fast muscle fiber growth was dominated by hypertrophy due to no increase in fiber number, increase in fibers with diameter > 200 μm and increased D mean of 95th percentile. Thus, having a subarctic and benthic lifestyle, wolffish seem to produce relatively large fibers and lower maximum fiber number similar to that of halibut, Dab and Notothenioid fishes.

5. Conclusion

There were no differences in growth rate or final weight when spotted wolffish were fed diets where up to 15% of the fishmeal and wheat was replaced with *N. oceanica*. The hematological analysis showed no indications of stress as an effect of the diets. A reduction in CF and HSI of the algae-fed fish as well as constant whole body lipid suggests a possible modulating effect on lipid metabolism. Level of linoleic acid (C18:2n-6) as well as PUFAs increased in both muscle, liver and whole body of all treatment groups, reflecting the use of about 50% plant-based ingredients in the diets. The ω-3 fatty acids were either conserved over the course of the experiment, or increased in the algae-fed groups, indicating a positive effect of *N. oceanica* on retention of PUFA's in spotted wolffish. There was no difference between groups in fast muscle cellularity, and growth in all groups were dominated by hypertrophy. The results indicate that wolffish might have a similar muscle fiber growth pattern as other fishes with a sub-arctic and benthic life history by producing relatively large fibers and a lower maximum fiber number.

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