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High protein requirements of juvenile Atlantic wolffish, *Anarhichas lupus*: Effects of dietary protein levels on growth, health, and welfare

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Abstract

The objective of the present study was to investigate the optimal dietary protein requirement and the effect of varying protein levels on the growth and health of juvenile, wild-caught Atlantic wolffish, *Anarhichas lupus*, a promising candidate for cold-water aquaculture diversification. Six iso-energetic (ca. 18.3 MJ kg^{-1}), fish meal-based experimental diets were formulated with crude protein levels ranging from 35% to 60%, with graded increments of 5% in a 12-week feeding trial in a recirculating aquaculture system (RAS). Weight gain, specific growth rate (SGR), and condition factor (K) were evaluated in response to dietary protein levels. Liver, muscle, and blood parameters were assessed for possible changes in protein and lipid metabolism and welfare. Overall growth was highly variable throughout the experiment on all diets, as expected for a wild population. The feed with highest in protein (60%) inclusion resulted in the highest growth rates, with an average weight gain of 37.4% ± 33.8% and an SGR of 0.31% ± 0.2% day^{-1}. This was closely followed by feeds with 55% and 50% protein inclusion with an average weight gain of 22.9% ± 34.8% and 28.5% ± 38.3%, respectively, and an SGR of 0.18% ± 0.3% day^{-1} and 0.22% ± 0.3% day^{-1}, respectively. Fish fed the high protein diets generally had increased hepatic lipid deposition (17%–18%) and reduced free fatty acid levels (3.1–6.8 μmol L^{-1}) in the plasma relative to fish that were fed the lower protein diets (35%–45%). No effects of diet were found on plasma protein levels or muscle protein content. Furthermore, stress parameters such as plasma cortisol and glucose levels were unaffected by diet, as were plasma ghrelin levels. Overall, these results suggest that a high protein inclusion in the diet for Atlantic wolffish is required to sustain growth with a minimum protein level of 50%.

KEYWORDS
aquaculture, Atlantic wolffish, growth, nutrition, welfare

1 | INTRODUCTION

Wolffish species of the genus *Anarhichas* consist of five different species with a benthic life style distributed in the North Atlantic Ocean, where two species the spotted wolffish (*Anarhinchas minor*) and Atlantic wolffish (*Anarhichas lupus*) have been proposed as a promising candidate species for cold-water marine aquaculture in North America and the Nordic countries (Falk-Petersen et al., 1999; Foss et al., 2004; Knutsen et al. 2019a, 2019b; Le François et al., 2002; Le François et al. 2021a). Although the spotted wolffish and Atlantic wolffish share similarities, most studies have focused on the potential of the spotted wolffish, with reportedly higher growth rates than the Atlantic wolffish (Moksness, 1990). Nevertheless, the geographic distribution and higher temperature optimum of the Atlantic wolffish make it an interesting option for local marine aquaculture diversification in the Nordic countries (Albertsson et al., 2012). Recent work in connection to farming of these two species has evaluated the potential of alternative feeds (Knutsen et al. 2019a, 2019b), growth potential (Árnason et al., 2019), reproductive management (Beirão et al., 2021; Beirão & Ottesen, 2018; Dupont Cyr et al., 2018; Le François et al. 2021b; Santana et al., 2020), and occurrences of xanthomatosis and nephrocalcinosis (Béland et al., 2020). Wolffish species display a docile behavior, are able to withstand high densities (Imsland et al., 2009; Tremblay-Bourgeois et al., 2010), and are tolerant to a wide range of abiotic stressors such as hypercapnia (Foss et al. 2003a), elevated ammonia levels (Foss et al. 2003b, 2003c), reduced salinity (Foss et al., 2001), as well as hypoxia and hyperoxia (Foss et al., 2002), making these species very resilient and promising for the diversification of cold-water aquaculture. Further, the relatively large first-feeding larvae will accept formulated feed (Foss et al., 2004).

However, some bottlenecks still remain for wolffish aquaculture. These include the lack of a well-defined macro-nutrient diet composition, and the lack of existing broodstock of Atlantic wolffish. To date, feed and growth studies have been limited to wild-caught individuals, where growth rates and feed acceptance can be highly variable, resulting in a cascade of physical, endocrinological, and physiological differences in other fish species studied (Devlin et al., 2020). An understanding of how key nutritional parameters affect growth and physiological performance is important for the selection of individuals that may thrive on formulated feeds in future aquaculture scenarios.

Proteins are essential for growth and synthesis of enzymes, hormones, and other metabolites, and it is crucial that their amount in feeds meets requirements for good growth, maintenance, and health status (Pond et al., 2004). In general, protein-rich feed (55%–62%) has been used in diets for spotted wolffish, both for first-feeding larvae and for juvenile stages (Foss et al., 2004), but good growth rates have also been obtained using feed with lower (45%–50%) protein contents (Moksness, 1990; Stefanussen et al., 1993). The protein requirement, that is, the minimum amount needed to meet amino acid requirements and achieve optimum growth and welfare should be the first nutritional parameter determined for novel aquaculture species and balanced so that health is not jeopardized. As protein is considered to have the highest cost of a diet it is crucial that protein is not provided in excess, to keep feed costs acceptable (FAO, 2022). Large differences exist among fish species in their requirements for protein and amino acids, likely due to differences in growth rate, feed intake, and the source of amino acids in the diet (Teles et al., 2020). In fish, excess dietary protein is used in intermediary metabolism and converted into glucose or lipids as energy deposits (Dabrowski & Guderley, 2002), which can then be mobilized during periods of stress, starvation, or malnutrition (Peres et al., 2014). From an environmental perspective, excess protein in aquaculture feeds can have a negative impact, as protein catabolism leads to increased ammonia excretion (Wilson, 2002) that may in turn affect the fish health and welfare and contribute to eutrophication (Cowey, 1995).

Feed intake (F), in turn, is driven by central and peripheral signals acting on appetite centers in the hypothalamus. One of the key peripheral signals affecting appetite regulation is ghrelin, indicated by most studies on fish to stimulate feed intake (Jönsson, 2013; Rønnestad et al., 2017). Plasma ghrelin levels increase during fasting and between meals when appetite increases, but less is known about the response of ghrelin to factors such as dietary composition (Rønnestad et al., 2017).

The aim of this study has been to estimate the protein requirement of juvenile Atlantic wolffish using a physiology-based approach. The main objectives were to investigate the effect of protein levels in fish meal-based diets on (1) growth (specific growth rate [SGR], weight gain, and condition factor, [K]); (2) appetite, assessed as feed intake and plasma ghrelin levels; (3) blood and plasma welfare and stress biomarkers (cortisol, glucose, and osmolality and oxygen-carrying capacity); (4) biochemical profile, measured as plasma levels of free fatty acids (FFA), L-amino acids and protein, and basic muscle and liver composition.

2 | MATERIALS AND METHODS

The holding of the fish and the experiment were performed in compliance with laws and regulations on animal experiments in Sweden (ethical permit no.: 208–2014), which are overseen by the Swedish Board of Agriculture and comply with the European Union legislation.

2.1 | Experimental diets and feeding

Six iso-energetic diets containing graded levels of crude protein were formulated (D1: 35.5%, D2: 40.4%, D3: 45.4%, D4: 50.4%, D5: 54.5%, and D6: 59.4%; Tables 1 and 2). Experimental feeds were produced at the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences (SLU, Uppsala, Sweden) by using standard experimental ingredients. Before feed production, the ingredients underwent chemical analysis to determine their composition. Dry matter was assessed by weighing the sample before and after desiccation at 103°C for 24 h. Ash content was determined by heating samples to 550°C for 24 h until there were no further changes in sample weight. Crude protein (CP) content was estimated

TABLE 1 Diet formulation (% *as is* basis) of the six experimental diets.

	Experiment diets					
Ingredients	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Fish meal (low temperature)	28.5	32.5	37	41	44	49.5
Fish oil	14.5	14	13	11	10	8.5
Wheat meal	10	10	11	10	10	3
Soybean meal	2	4	6	6	6	6
Casein	5	4.5	2	2	1	1
Wheat Gluten	3	5	9	12	16	18
Gelatine	6	6	6	6	6	6
Carboxymethyl cellulose binder	1	1	1	1	1	1
Mineral premix	2	2	2	2	2	2
Methionine	0.5	0.4	0.3	0.2	0.1	0
Lecithin	1	1	1	1	1	1
Corn starch	17	10	5	2	0	0
Cellulose	9.1	9.3	6.4	5.6	2.7	4
Total	100	100	100	100	100	100

Note: Diet 1 (D1): 35.5% protein content, Diet 2 (D2): 40.4% protein content, Diet 3 (D3): 45.4% protein content, Diet 4 (D4): 50.4% protein content, Diet 5 (D5): 54.5% protein content, Diet 6 (D6): 59.4% protein content. From here on, experimental diets will be referred to by their acronyms given in this table legend.

TABLE 2 Proximate composition of the six experimental diets (D1–D6).

Composition (g per 100 g of dry matter)	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Dry matter	95.3	94.9	94.6	94.4	94.4	94
Ash	5.3	6.2	7.2	7.9	8.5	9.4
Crude protein[a]	35.5	40.4	45.4	50.4	54.5	59.2
Crude lipid[b]	16.2	15.9	15.3	13.5	12.7	11.4
Gross energy MJ/kg[c]	18	18.1	18.4	18.3	18.6	18.4
Carbohydrate	23.9	18.2	12.5	8.6	4.3	0.3
Total phospholipid	1.0	1.0	1.0	1.0	1.0	1.0
Calcium	1.1	1.2	1.2	1.4	1.4	1.5
Essential amino acids (%)						
Arginine	1.4	1.7	2.0	2.3	2.5	2.8
Histidine	0.6	0.8	0.9	1.0	1.1	1.2
Isoleucine	1.5	1.7	1.9	2.2	2.4	2.7
Leucine	2.1	2.4	2.7	3.1	3.4	3.8
Lysine	2.7	2.9	3.3	3.8	4.1	4.6
Methionine	1.3	1.3	1.3	1.3	1.3	1.3
Phenylalanine	1.2	1.4	1.6	1.9	2.1	2.4
Threonine	1.0	1.2	1.3	1.4	1.5	1.7
Valine	1.5	1.7	1.9	2.1	2.3	2.6

[a]According to Kjeldahl (N*6.25).
[b]According to Schmid-Bondzynski-Ratzlaff.
[c]Using bomb calorimetry.

from the total nitrogen content, calculated as the nitrogen content multiplied by 6.25, and the total nitrogen was quantified using the Kjeldahl method with a 2020 digester and a 2400 Kjeltec Analyzer unit (FOSS Analytical A/S, Hillerød, Denmark). Crude lipid (CL) content was determined through hydrolyzation using a 1047 Hydrolysing Unit and a Soxtec System HT 1043 Extraction Unit (FOSS Analytical A/S). Gross energy was assessed using isoperibol bomb calorimetry (Parr 6300, Parr Instrument Company, Moline, IL, USA).

Amino acid profiles were analysed at a certified laboratory (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden). Amino acids were quantified using high-performance liquid chromatography.

The feed ingredients were then mixed with a mixture of hot water and gelatine (binder), added drop-wise to reach the desired consistency. The resulting paste was processed through a meat grinder (Nima Maskinteknik AB, Örebro, Sweden) to produce 1.5 mm pellets that were placed in a steam oven (105°C, for 1 min) to increase gelatinization. Pellets were then oven-dried (using a forced-air oven; 45°C, 24 h until there were no further changes in mass) in a drying cupboard. The dry feed used in the experiment was stored in airtight containers at 4°C.

2.2 | Fish origin and facilities

Juvenile Atlantic wolffish were reared from a cohort of wild-caught fertilized eggs, collected during a bottom trawl survey by the Icelandic Marine and Freshwater Research Institute (Hafnarfjörður, Iceland) off the northwest coast of Iceland. Eggs were hatched in April 2017 and fish kept in the flow-through system of the Aquaculture Research Station of Grindavík (Iceland), until they reached 7 g. They were subsequently transferred to the experimental facilities of the University of Gothenburg (Gothenburg, Sweden) in June 2017, where they were kept in 110-L round tanks with recirculating, oxygenated seawater at 10°C. Fish were fed a commercial diet (Skretting Amber Neptun, grade 1.0 from 7 to 20 g and Skretting Amber Neptun grade 2.0 from 20 to 40 g) until there was apparent satiation on a daily basis up until the start of the experiment.

In November 2017, 360 fish were anesthetized (MS-222, Finquel, Argent Chemical Laboratories, Redmond, USA, [0.16 g L^{-1}]) and individually tagged intraperitoneally with passive integrated transponder (PIT) tags (12 mm, Biomark, Boise, USA). The fish were then measured for weight and length. The initial mean weight of the fish was 40 ± 8 g, and weights and lengths were recorded every 3 weeks after tagging. Fish were randomly distributed into 18 experimental tanks (20 L; $n = 20$ per tank), in triplicate. The water system was a recirculating aquaculture system (RAS), with a temperature of 10°C and a 32 ‰ salinity, a photoperiod set at 12:12 L:D, and a light intensity of 80 lux. The water flow rate was 0.5 L min^{-1}. Experimental tanks were fitted with a 24-h online monitoring system connected to a temperature alarm function (Sensdesk, HW group, Prague, Czech Republic). Fish density was set at 12.5 kg m^2, well within the optimum density for wolffish species of this weight class (below 30 kg m^2) (Le François et al., 2013; Tremblay-Bourgeois et al., 2010). Fish were allowed to acclimatize to these conditions for 7 days. During this period fish were fasted for 3 days before being reintroduced to the commercial diet. Fish were weaned onto experimental diets from the commercial feeds by replacing 5% of commercial feed with portions of experimental feed at steady intervals over a 3-week period. After the weaning period, fish were fed one of the six experimental diets (in triplicate) for 12 weeks, at 0.5% body weight per day. This was close to satiation from observations and previous feed consumption of the commercial diet before the feeding trial, in agreement with previous observations for spotted wolffish (Foss et al. 2003a, 2003b, 2003c; Imsland et al., 2009). The feed was administered by hand twice daily (09:00 and 16:00). Thirty minutes after each feeding, uneaten pellets from each tank were collected and counted to determinate the daily feed intake. Environmental parameters (temperature, °C; oxygen, mg L^{-1}; and salinity, ‰) were recorded daily using a VWR portable Multimeter pHenomenal MU 6100 H (VWR international, Radnor, USA). Inspection for potential dead fish was carried out daily. Dead fish were removed from the system and registered.

2.3 | Growth and biometrics

Fish size was determined at weeks 0, 3, 6, 9 and 12 by recording total body length (BL; cm) and body weight (BW; g) of individual fish that were fasted for 24 h prior to measurements. Weight gain (%) and SGR for body were calculated using the following formulas:

$$\text{Weight gain}(\%) = [(BW_F - BW_I)/BW_I] \times 100, \text{where } BW_I \text{ and } BW_F \text{ are the initial and final body weights, respectively.}$$

$$SGR_W = 100 \times [\ln(BW_F \times BW_I)]/D, \text{where } BW_I \text{ and } BW_F \text{ are the initial and final body weights respectively(g).}$$
D is the number of treatment days.

$$\text{Condition factor(K) was calculated as } K = \left(BW_F/BL_F{}^3\right) \times 100,$$
where BW_F and
BL_F are the final body weight(g) and length, respectively(cm).

Feeding intake (F, % biomass in tank) was calculated on a tank basis for each diet with the following formulas:

$$F = [(C_T/W) \times 100],$$
where C_T is daily feed consumption(g dry weight consumed) and
W is the mean biomass in that tank(g).

2.4 | Final sampling, tissues, hematology, and plasma parameters

After 12 weeks, four fish from each tank ($n = 12$ per diet) were euthanized with an overdose of Aquacalm (methomidate, Syndel, USA, 0.1 g L^{-1}) followed by a sharp blow to the head, and then weighed and measured. Liver and mesenteric fat were dissected and weighed. The liver weight (LW, g) was used to calculate the hepatosomatic index (HSI) = [(LW/BW_F) × 100]. The mesenteric fat (MF_W, g), mainly surrounding the intestine, was weighed to calculate mesenteric fat index (MFI) = [(MF_W/BW_F) × 100]. A small piece of tail muscle (i.e., 1 cm^2) was removed, frozen, and later homogenized using a meat mincer and subsequently analysed for muscle protein content.

Blood samples were taken from the caudal vessels of the four fish from each tank ($n = 12$ per diet), using a 1-mL heparinized syringe with a 25-gauge needle. Hemoglobin concentration ([Hb]) was determined using a handheld hemoglobin analyser (HemoCue 201+, Ängelholm, Sweden) and values corrected for fish blood (Clark et al., 2008). Hematocrit (Hct) was assessed in duplicate by drawing blood into a heparinized capillary tube that was sealed with critoseal, and then centrifuged for 5 min in a micro centrifuge (Thermoscientific Heraeus Pico 170, Thermo Fisher Scientific, Waltham, USA). The packed cell volume was read using a Hawksley reader (Hawksley & Sons Ltd., Lancing, UK) and recorded as a percentage of the total blood volume. The mean corpuscular hemoglobin concentration (MCHC, g Hb dL^{-1}) was calculated as (MCHC) = [Hb]/Hct × 100. The remaining blood was immediately centrifuged using a tabletop spinner (Thermoscientific Hareus Pico 17, Thermo Fisher Scientific) for 5 min, and the obtained plasma was collected and stored at −80°C for later analyses.

2.5 | Plasma biochemistry

Plasma glucose (mmol L^{-1}), FFA, and free L-amino acid (μmol L^{-1}) and protein levels (mg mL^{-1}) were measured using commercial colorimetric kits (Gahk20, Mak044, Mak002, and BCA 1, Sigma Aldrich, Saint Louis, USA; Instruchemie, Delfzijl, The Netherlands). Plasma osmolality (mOsmol L^{-1}) was measured using a cryoscopic osmometer with 50 μL as a sample volume (Advanced Model 3320 Micro-Osmometer4, Advanced Instruments Inc., Norwood, USA).

Plasma cortisol (nmol L^{-1}) was assessed using a radioimmunoassay according to Young (1986) using modifications reported in Sundh et al. (2011). Plasma levels of active, acylated ghrelin (pmol L^{-1}) were analysed following the n-ghrelin protocol established by Hosoda et al. (2000), modified by Jönsson et al. (2007), with the exception that plasma was not extracted before measurements and iodinated human ghrelin (NEX388010UC, Shelton, PerkinElmer, USA) was used as a tracer. Anti-rat ghrelin [1–11] antisera, which specifically recognizes the conserved n-octanoylated Ser3 epitope on ghrelin, was used at a dilution of 1:1,000,000 (gift from Dr. Hiroshi Hosoda, Japan). Synthetic rainbow trout acylated ghrelin (Peptide Institute, Osaka, Japan) was used for the standard curve. All samples were assayed in duplicate. The ghrelin RIA was validated for Atlantic wolffish with a test of parallelism using a serial dilution (1:2) of plasma.

2.6 | Proximate composition analysis of liver and muscle

For analysis of proximate composition (protein and lipid, % wet weight), tissues ($n = 12$ per diet) were homogenized. Total protein content was measured using a LECO nitrogen analyser (TruMac-N, LECO Corp., Saint Joseph, USA), with a 5.58 conversion factor (Mariotti et al., 2008). Approximately 2 g of samples was subjected to nitrogen measurement, with two replicates. Total lipid content (%) was analysed using a gravimetric method as described by Lee et al. (1996), modified by Powell et al. (2017).

2.7 | Statistical analysis

Statistical analysis was performed using SPSS (SPSS, Chicago, USA). All parameters were analysed and compared within the discrete treatments, and survival is expressed as a percentage. Assumptions of normality and homogeneity of variance were assessed using the Shapiro–Wilk test and Levene's test, respectively, followed by individual inspection of data distribution curves. Variables failing these assumptions were $\log_{10}$ transformed. A one-way nested ANOVA was used to compare the means among the dietary groups, with tanks nested in the analysis to identify potential tank effects. When a significant p-value was detected ($p < 0.05$), multiple comparisons were made among groups with the Tukey post hoc test. Kruskal-Wallis tests were used for variables displaying heterogeneous variance for which transformations were unsuccessful (glucose, osmolality, and cortisol). Dunn's post hoc method was used to analyse medians of significant p-values from the Kruskal-Wallis test. All data are presented as means ± standard deviation of the mean (SD).

3 | RESULTS

3.1 | Observations

All feed pellets were negatively buoyant and subsequently appeared physically stable during 24 h immersion. The wolffish inspected and accepted all feeds soon after introduction, but less so in the D3 treatment (45% protein). Mortality among the six treatments was not significantly affected by diet or tank (Table 3; nested one-way ANOVA, $p = 0.89$). Toward the end of the experiment (11–12 weeks) some fish in all treatments developed symptoms of exophthalmia. This was random among all the dietary treatments, and these fish were removed from the experiment and do not contribute toward statistics or mortality.

3.2 | Growth and biometrics

The diet groups differed significantly in weight gain and SGR, with a general increase from increased protein content in the diet (Figure 1). Fish fed D4 and D6 had a significantly higher average SGR (0.19%–0.31% day^{-1}) compared to fish fed D1–D3 (0.01%–0.07% day^{-1}; Figure 2a; ANOVA, $p < 0.01$). In tandem with SGR, fish fed D4 and D6 displayed significantly higher weight gain (23%–38%), compared to fish fed D1–D3 (3%–10%; Figure 2b; ANOVA, $p < 0.01$). No significant differences were detected for SGR or weight gain between the D4–D6 diets. K was elevated in D6, but only significantly higher relative to fish fed D1–D3 (Figure 2c; ANOVA, $p < 0.01$). Feeding rate varied significantly among fish in the dietary treatments; fish fed D1 and D4–D6 had a significantly higher feeding rate relative to fish fed D2–D3 (Table 3; ANOVA, $p < 0.01$). The nested ANOVA showed a tank effect occurring on daily feeding rate ($p < 0.01$), whereas no tank effect was detected for K ($p = 0.61$). No significant differences were found in HSI or MFI among the different diet groups (ANOVA, $p = 0.64$ and $p = 0.07$ respectively; Table 3).

Diet	BW_I (g)	BW_F (g)	F	HSI	MFI	Survival (%)
D1	41.2 ± 8.8	45.5 ± 14.7	0.3 ± 0.0^{b}	3.1 ± 0.6	0.3 ± 0.1	91.6 ± 4.7
D2	40.3 ± 8.4	46.1 ± 11.9	0.2 ± 0.0^{a}	2.8 ± 0.0	0.4 ± 0.1	91.6 ± 2.3
D3	40.6 ± 7.5	41.5 ± 10.4	0.2 ± 0.0^{a}	2.8 ± 0.2	0.3 ± 0.1	96.6 ± 2.3
D4	40.2 ± 9.9	54.8 ± 20.9	0.3 ± 0.0^{bc}	3.1 ± 0.1	0.4 ± 0.0	95.0 ± 4.1
D5	40.1 ± 9.4	53.4 ± 16.7	0.3 ± 0.0^{b}	2.9 ± 0.1	0.5 ± 0.1	91.6 ± 6.2
D6	40.7 ± 8.7	56.3 ± 16.3	0.4 ± 0.0^{c}	3.4 ± 0.4	0.5 ± 0.1	95.0 ± 7.0

Note: All parameters show mean values ± SD ($n = 12$). Letters show significance groups from ANOVA and Tukey post hoc test.

TABLE 3 Initial body weight (BW_I), final body weight (BW_F), feed intake (F), hepatosomatic index (HSI), mesenteric fat index (MFI), and survival of Atlantic wolffish fed different diets based on means.

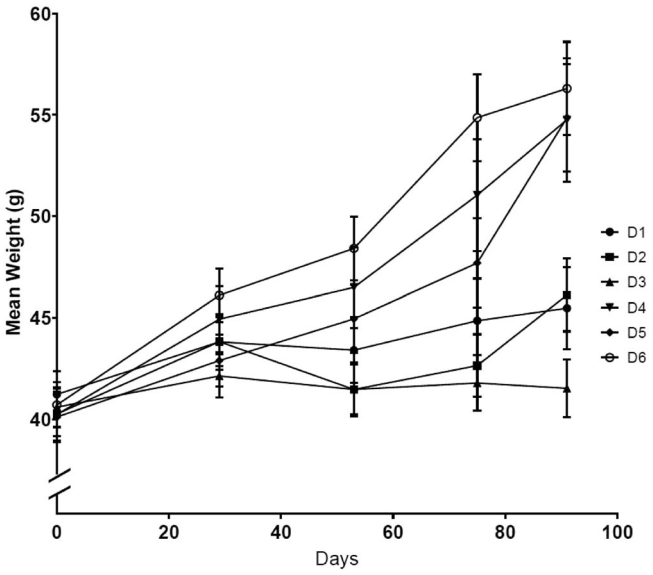

FIGURE 1 Average body weight (g) among tanks for experimental diets D1–D6. Data presented as means ± SD ($n = 3$).

3.3 | Hematology and plasma parameters

Nutritional plasma parameters are given in Table 4. Fish fed D6 had FFA plasma levels of 3.1 μmol L^{-1}, significantly less than fish fed D1 (11.8 μmol L^{-1}; ANOVA, $p < 0.05$). Free L-amino acids and plasma protein levels ranged between 76.5–82.7 μmol L^{-1} and 8.9–10.4 mg mL^{-1}, respectively, and did not differ significantly among the dietary groups (ANOVA, $p = 0.34$ and $p = 0.91$, respectively; Table 4). Plasma glucose levels ranged between 2.1 and 2.9 mmol L^{-1} between the different dietary treatments, showing no differences between diets (Kruskal-Wallis, $p = 0.53$). Hematology and other plasma parameters are presented in Table 5. There were no significant differences among the dietary treatments for Hct (22.0%–25.5%, ANOVA, $p = 0.34$), Hb (3.4–3.9 g dL^{-1}, ANOVA, $p = 0.37$), and plasma osmolality (Kruskal-Wallis, $p = 0.91$; Table 5). Cortisol levels did not differ significantly (Kruskal-Wallis, $p = 0.11$) among the dietary groups, ranging between 5.8 and 15.7 nmol L^{-1}. Ghrelin levels were also unaffected by dietary treatments, ranging between 2.5 and 4.2 pmol L^{-1} (ANOVA, $p = 0.25$).

3.4 | Proximate composition parameters

The nutritional characteristics of the whole fish, liver, and muscle are presented in Table 4. There were no differences in crude protein content of muscle among the diets (ANOVA, $p = 0.62$). Muscle lipid content was not affected by the different diets, with values ranging between 1.8% and 3% wet weight (ww) across D1–D6 (ANOVA, $p = 0.62$; Table 4). Fish fed D4–D6 had significantly higher liver lipid content (17.5%–18.6%) compared to fish that were fed D1 (14.8%; Table 4; ANOVA, $p < 0.05$).

4 | DISCUSSION

4.1 | Growth

Overall, the highly variable growth and feeding rates observed suggest that there is a large genetic variability in these wild-caught fish. Although problematic within the context of a feeding trial, this indicates a large potential for breeding selection for this species.

The present study suggests that Atlantic wolffish juveniles grow best on feed with high dietary protein content (50%–60%), confirming an earlier work that indicated a dietary protein requirement above 50% (Foss et al., 2004; Stefanussen et al., 1993; Strand et al., 1995). Carbohydrates are routinely used in aquafeeds to replace protein. However, poor starch digestibility can also cause growth retardation in a variety of fish species (Kamalam et al., 2017; Maas et al., 2020; Stone, 2003). The low protein diets in the present study contained a carbohydrate content in the range of (12%–23%, D3–D1). It is indeed possible that poor carbohydrate utilization of fish on these diets caused their poorer growth performance compared to fish on the higher protein diets, which contained much less (0.3%–8%) carbohydrate (D6–D4). However, carbohydrate digestibility has not been extensively investigated within fish nutrition and differs widely among fish species (Maas et al., 2020). For novel species such as Atlantic wolffish, the effects of high carbohydrate variability within feeds are still unknown. However, reduced growth rates of juvenile Atlantic wolffish were previously observed only when carbohydrate inclusion levels reached 28%–29% (Stefanussen et al., 1993), suggesting that starch levels used in the present study should not have had a negative effect on growth.

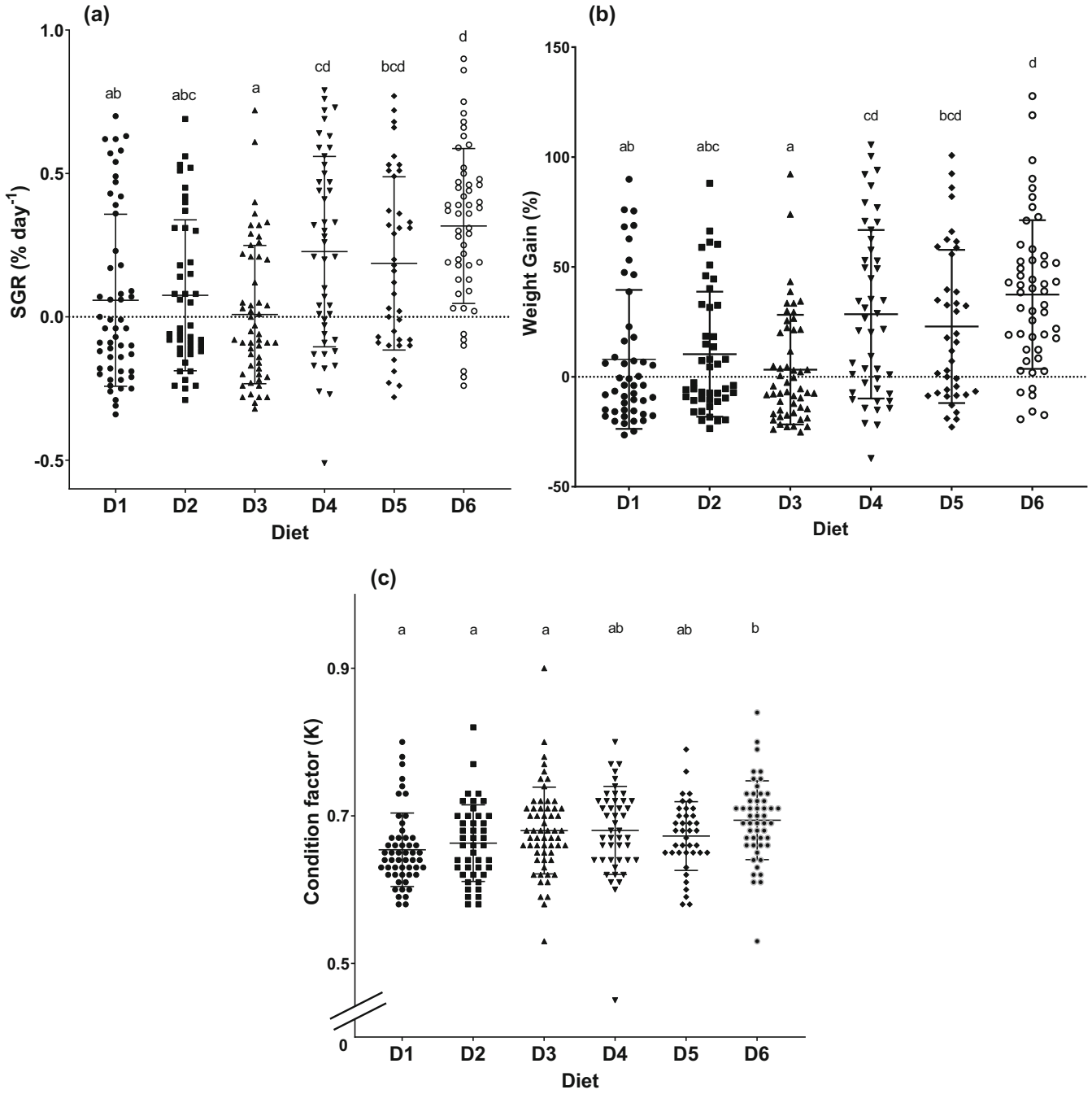

FIGURE 2 (a) Individual specific growth rate in weight (% day^{-1}; horizontal lines showing mean ± SD). (b) Individual weight gain (%; horizontal bars showing mean ± SD). (c) Individual condition factor (K; horizontal bars showing men ± SD). Letters show significance groups from ANOVA and Tukey post hoc test ($n = 3$).

For the fish on the higher protein diets in the present study, the SGR (0.2%–0.3% BW day^{-1}) and weight gain (25%–35%) is lower than previously reported for this and other fish species. Stefanussen et al. (1993) reported SGR of 0.67% BW day^{-1} in 40 g Atlantic wolffish, fed a diet containing 54% protein. McCarthy et al. (1998) reported SGR of 0.91% BW day^{-1} in 25 g Atlantic wolffish, and Árnason et al. (2019) reported SGR as high as 1% BW day^{-1} in 20 g fish. Individual growth analysis, from the pit-tagging, revealed that some individuals of all diet groups either did not gain weight or even lost weight, during the experiment. This suggests that marked individual variability in the ability to wean successfully onto the diets is the cause of the relatively low average growth rates. On the contrary, all diet groups also had individuals with relatively high growth rates, well in line with previous data, as discussed earlier. The large individual variation in SGR observed in the current study strongly indicates that the selection for faster growth might rapidly yield positive results in a breeding programme for Atlantic wolffish. This has previously been accomplished for other species such as the Atlantic salmon, *Salmo*

TABLE 4 Liver fat, muscle fat, protein content of muscle, and plasma levels of glucose, free fatty acids (FFA), free amino acids (AA), and protein of Atlantic wolffish fed six experimental diets (D1–D6).

Diet	Liver fat (% ww)	Muscle fat (% ww)	Muscle protein (% ww)	Plasma glucose (mmol L^{-1})	Plasma FFA (μmol L^{-1})	Plasma AA (μmol L^{-1})	Plasma protein (mg mL^{-1})
D1	14.8 ± 1.4[a]	2.9 ± 0.8	16.6 ± 0.7	2.8 ± 1.3	11.8 ± 6.7[a]	81.3 ± 13.8	10.4 ± 2.3
D2	17.1 ± 1.9[ab]	3.0 ± 0.8	16.0 ± 1.2	2.3 ± 1.7	10.0 ± 8.3[ab]	82.7 ± 18.3	8.9 ± 1.7
D3	16.5 ± 1.6[ab]	2.0 ± 0.5	16.3 ± 0.8	2.6 ± 1.7	6.7 ± 6.1[ab]	79.2 ± 10.4	9.3 ± 1.3
D4	18.6 ± 2.8[b]	1.8 ± 0.5	16.4 ± 0.5	2.1 ± 0.7	5.2 ± 5.7[ab]	77.1 ± 13	10.5 ± 1.5
D5	17.9 ± 1.8[b]	2.8 ± 0.8	16.2 ± 1.0	2.3 ± 0.9	6.8 ± 4.0[ab]	79.7 ± 11	10.4 ± 2.1
D6	17.5 ± 2.3[ab]	2.6 ± 0.8	16.9 ± 0.5	2.9 ± 1.5	3.1 ± 1.8[b]	76.5 ± 8.3	8.9 ± 0.5

Note: All data are presented as means ± SD ($n = 12$). Letters show significance groups from ANOVA and Tukey post hoc test.

TABLE 5 Plasma ghrelin and cortisol levels, osmolality, Hb, Hct, and MCHC of Atlantic wolffish fed different diets (D1–D6).

Diet	Plasma ghrelin (pmol L^{-1})	Plasma cortisol (nmol L^{-1})	Plasma osmolality (mOsmol L^{-1})	Hb (g dL^{-1})	Hct (%)	MCHC (g Hb dL^{-1})
D1	4.2 ± 1.7	15.0 ± 11.7	387.4 ± 35.9	3.9 ± 0.6	25.4 ± 2.4	15.2 ± 1.8
D2	3.2 ± 3.2	8.2 ± 9.3	375.5 ± 18.8	3.6 ± 0.4	22.6 ± 2.5	15.9 ± 1.0
D3	2.7 ± 2.2	12.0 ± 11.8	368.0 ± 34.8	3.4 ± 0.6	22.0 ± 3.0	15.6 ± 1.4
D4	3.6 ± 2.1	7.7 ± 9.3	380.1 ± 20.4	3.8 ± 0.7	24.0 ± 1.8	15.3 ± 1.5
D5	2.5 ± 1.2	15.7 ± 14.1	380.6 ± 19.8	3.6 ± 0.3	23.7 ± 1.9	15.4 ± 0.5
D6	3.3 ± 1.3	5.8 ± 5.0	383.7 ± 26.8	3.5 ± 0.6	25.5 ± 3.8	15.4 ± 1.3

Note: All data are given as means ± SD ($n = 12$). Letters show significance groups from ANOVA or Kruskal-Wallis (osmolality).

salar, where growth rate heritability is high, resulting in 10%–15% improvement in growth rate per generation (Gjedrem, 2000). Fewer individuals showed these growth rates in the low protein diet groups, but these were still comparable to the highest growth performances observed in fish on the higher protein diets.

Growth in fish is highly dependent on feed consumption, and in the present study, there was a general reduction in the daily feed intake of low protein diets, except for the D1 diet. The individual data on growth rates, together with the consumption data, suggest the potential establishment of feeding hierarchies. Some dominant individuals on the lower protein diets could have compensated for the lower protein inclusion by increasing their consumption rate, as seen in salmonids (Azevedo et al., 2004; Geurden et al., 2006).

4.2 | Proximate composition

In the present study, liver lipid content increased with dietary protein content in the diet, between 14% and 18% from D1 to D6. The differences in liver lipid content among dietary groups may reflect a shift to a catabolic state, where energy needs to sustain growth for fish on the lower protein diets were met by mobilization of liver energy reserves. Similar results have been reported for European seabass, *Dicentrarchus labrax* (Peres et al., 2014), and sea bream, *Sparus aurata* (Peres et al., 2011), during fasting periods.

Teleosts have various strategies for storing lipids as energy reserves. Commonly, fish are classified as either "lean" or "fatty" to illustrate two main strategies. "Lean" fish (e.g., Atlantic cod, *Gadus morhua*) are characterized by low muscle fat (1%–2%) and a large, fatty liver, which is the main site for energy storage (dos Santos et al., 1993; Houlihan et al., 2008). In contrast, "fatty" fish (e.g., Atlantic salmon) have relatively small and less fatty liver, but high mesenteric and/or muscle lipid content, representing the major energy stores (Houlihan et al., 2008). The Atlantic wolffish appears to be somewhat intermediary regarding the "lean" and "fatty" categories. Hepatic lipidosis was linked to high fat content of feed (15%–18%) by Chabot et al. (2012) and discussed by Le François et al. (2021a). The present data show that the liver has a high lipid content, but is relatively small with an HSI around 3%, compared with a typical "lean" whitefish species such as the Atlantic cod with an HSI of 6%–7% (Houlihan et al., 2008). In the present study, there were no differences in HSI among the dietary groups, whereas in Atlantic cod, HSI is affected by dietary variations in both protein and lipid (Árnason et al., 2010; Grisdale-Helland et al., 2008). The muscle lipid content of the Atlantic wolffish was relatively low (1.8%–3.0%), compared with that of a "fatty" species such as the Atlantic salmon (ca. 10%) (Houlihan et al., 2008) yet higher than that of a "lean" species such as the Atlantic cod (ca. 0.5%) (Houlihan et al., 2008). In the present study, neither the protein nor lipid content of the muscle was affected by diet, suggesting that different protein levels in feed did not affect the nutritional quality of the fillets. In concordance with this, studies on spotted wolffish show that muscle crude lipid does not change with levels of the marine algae, *Nannochloropsis oceanica*, added to supplement protein in the diet (Knutsen et al. 2019a). It is clear from these results that more research is warranted with regard to lipid storage and lipid metabolism in wolffish.

4.3 | Plasma biochemistry

In the present study, the only observed effect on plasma biochemistry was a decrease in FFA levels with increasing levels of protein in the diet. The increased levels of FFA in the lowest protein diet group (D1), closely followed by D2 and D3, support the idea that an elevated rate of lipolysis in these fish occurs to sustain growth. Alternatively, high protein diets, especially D6, promote liver lipogenesis. In the European eel (*Anguilla anguilla*), starvation induces a significant increase in plasma FFA levels as a result of an increased hepatic lipolysis (Larsson & Lewander, 1973). Di Marco et al. (2008) suggested that plasma FFA may be produced from hepatic tissue and mesenteric fat, rather than from the breakdown of triglycerides in the blood in the European seabass. The different lipid storage strategies of different teleost species likely lead to a varying importance in the breakdown and release of FFA from fat depots and require further investigation.

Plasma protein levels in fish are often associated with nutritional and physiological status (Congleton & Wagner, 2006; Řehulka et al., 2005), showing stable levels in well-nourished animals and decreased levels under fasting conditions (Peres et al., 2014). Often, reduced plasma protein levels occur as a consequence of amino acid oxidation or peripheral proteolysis (Di Marco et al., 2008). However, no effects of dietary protein content on plasma protein levels or L-amino acid levels were observed in the present study. This indicates that although the fish grew less at lower protein levels, they managed to maintain a good protein balance, both in the plasma and in the muscle. Therefore, the lower growth rates observed in D1–D3 may be attributed to the relatively high cost of maintaining the overall protein homeostasis with limited resources from the diets.

The fact that the treatments did not affect plasma osmolality and cortisol suggests that the fish were not stressed by the experimental conditions and that hyposmoregulatory ability was maintained as plasma osmolality was within the range previously found for spotted wolffish (Foss et al., 2001; Knutsen, Johnsen, et al., 2019a; Le François et al., 2013). A study on Atlantic salmon has shown that ghrelin levels may change in response to differences in feed intake and dietary lipid and/or energy content, which may imply nutrient-dependent effects on ghrelin release (Johnsen et al., 2011). However, in the current study, plasma ghrelin levels were similar among groups with different feed intake, indicating that dietary protein content does not influence ghrelin levels.

4.4 | Potential for aquaculture

Despite a relative apparent heterogeneous/slow growth, as expected from a wild-caught population, Atlantic wolffish have several important advantages for farming such as the apparent ability to consume dry feed directly after hatching, which makes the use of commercially expensive live, larval feed unnecessary (Foss et al., 2004). To date, there exists no commercial fishery for this species, with the wild product fetching relatively high market prices. Árnason et al. (2019) demonstrated the growth potential for Atlantic wolffish with growth hormone implants, which increased weight gain with 30% throughout the experimental period in a 3–11°C temperature range. This species thus clearly demonstrates potential for growth promotion through domestication and selective breeding, and as the current study shows, there is a large growth potential on an individual basis. Coupled with this, growth rates in Atlantic wolffish are significantly influenced by dietary protein, suggesting that both increased growth and higher growth efficiency will be possible at 10–11°C as the optimal dietary formulations become better known and as rearing conditions and domestication across different generations are optimized. With an optimized essential amino acid profile, dietary proteins will be required in lower quantities and will be utilized more efficiently for growth in future scenarios. Thus, dietary protein, energy ratio, digestibility, and amino acid profile need to be optimized to maximize growth performance and to decrease feed costs and the negative environmental impacts of feeding.

5 | CONCLUSIONS

Protein requirements for growth and health of juvenile Atlantic wolffish appear to be similar as for other marine species (50%–60%). This study suggests that a protein level of 50% does not negatively impact physiology or welfare parameters, while maximum growth is observed in Atlantic wolffish. Thus, this protein inclusion level could be a good trade-off between optimal growth, good welfare, and a cost-efficient and sustainable diet, ensuring the resilience of the aquaculture sector.

AUTHOR CONTRIBUTIONS

James Hinchcliffe conceived the idea of the study, and all authors were involved in the planning and design. James Hinchcliffe, Jonathan A.C. Roques, Josefin Roos, and Ida Hedén were involved in data generation. James Hinchcliffe, Josefin Roos, and Markus Langeland formulated and produced the feeds used in this study. James Hinchcliffe performed the data analysis. James Hinchcliffe, Jonathan A.C. Roques, and Elisabeth Jönsson were involved in the manuscript preparation. Kristina Sundell, Björn Thrandur Björnsson, and Elisabeth Jönsson were responsible for funding the study. All authors have reviewed and accepted the final version of this manuscript.

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