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Use of a mixture design to optimize dietary macronutrients for large turbot (*Scophthalmus maximus* Linnaeus, 1758)

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Abstract

Aim of study: Studies on the dietary needs of turbot fish (*Scophthalmus maximus* Linnaeus, 1758) have largely focused on the juvenile stage; however, there are not many on the larger (300–500 g) species. The purpose of this experiment was to determine the ideal dietary levels of protein, fat, and carbohydrate for large turbot.

Area of study: Demre, Antalya, Türkiye.

Material and methods: A three-component mixture design model was created to adjust the quantities of dietary protein between 45.6% and 63.4%, carbohydrates between 4.9% and 30.5%, and fat between 5.6% and 17.7%. The components of the model were fish meal (FM), fish oil (FO), and wheat flour (W). Fish initially weighing 301.6±0.1 g on average were fed 14 different diets for 10 weeks. The ideal dietary macronutrient levels were estimated by examining the prediction profiler at the highest desirability based on the variables that were selected to maximize final weight, daily growth coefficient, protein efficiency ratio, nitrogen and energy retentions, and minimize feed conversion ratio, nitrogen and carbon losses.

Main results: The optimal diet formulation yielded the highest desirability of 0.87 for all selected responses and resulted in dietary inclusion levels of FM, W and FO as 63.6%, 20.8%, and 9.4%, respectively. The proposed optimal nutrient concentrations for large turbot (growing from 300 to 500 g) are 54% protein, approximately 17% lipid, and 15.8% carbohydrate on dry matter basis.

Research highlights: The mixture design successfully allowed us to estimate the optimum levels of dietary protein, lipid and carbohydrate for large turbot.

Additional key words: *Scophthalmus maximus*; aquaculture; nutrient requirements; diet formulation

Abbreviations used: C (carbon); CF (condition factor); DFI (daily feed intake); DGC (daily growth coefficient); FCR (feed conversion ratio); FM (fish meal); FO (fish oil); FW (final weight); HSI (hepato-somatic index); IW (initial weight); PER (protein retention efficiency); MBW (metabolic body weight); N (nitrogen); PCA (principal component analysis); VSI (viscera somatic index); W (wheat flour).

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Introduction

Aquaculture has become a mainstream food supply system, with more than 600 aquatic animal species being farmed worldwide (Troell et al., 2014; Naylor et al., 2021). To ensure sustainable production of these species, nutritionally complete diets must be provided throughout their developmental stages (Lupatsch, 2009). To achieve this, more precise estimations of the nutritional requirements of each species at different developmental periods are necessary to establish species-specific diets (Glencross et al., 2007; Lupatsch, 2009; Hardy et al., 2022). While acceptable quality standards for nutritional and pellet properties in aquaculture are provided by current knowledge and practice, they are still far from ideal (Turchini et al., 2019; Kaushik & Schrama, 2022). Diet formulations and ingredient selections in modern aquaculture diets are based on dietary protein, lipid, and carbohydrate concentrations and their energetic levels (Phan et al., 2019; Turchini et al., 2019). An optimum balance between dietary protein, lipid, and carbohydrate requirements of fish species across the growing stages should be achieved by conducting experiments that simultaneously consider the selected responses using cost-effective and labor-efficient experimental methods (Hamre et al., 2003; NRC, 2011; Turchini et al., 2019).

Using a minimum experimental unit, the mixture design recommends optimal dietary amounts and permits simultaneous screening of a wide range of nutrient compositions within predetermined limitations (Hamre et al., 2003, 2022; Vielma et al., 2003). Identifying important nutrients and determining the species' estimated allowed quantities of those nutrients is the first step (Vielma et al., 2003; Ruohonen et al., 2007; Enyidi et al., 2017), followed by choosing an appropriate design type (simplex, centroid, or axial) considering the number of components and constraints using statistical software (Montgomery, 2017). The software generates experimental runs based on constraints and design type, which are then tested to gather the necessary data. A statistical analysis of the data reveals important effects of individual nutrients and their interactions, which ultimately helps interpret and optimize the nutrient combination for best performance (Vielma et al., 2003; Ruohonen et al., 2007; Khuri & Mukhopadhyay, 2010; Enyidi et al., 2017). Therefore, this method can be used to develop nutritionally complete, environmentally friendly, and economically cost-effective diets for aquaculture species, including turbot.

Turbot (*Scophthalmus maximus* Linnaeus, 1758) is an economically important fish, and its production under aquaculture conditions has increased to meet the demand because its wild stocks cannot meet the market's needs. The world turbot aquaculture production was 77,110 tons in 2019 (FAO, 2022). Despite a significant increase in production, there is still an information gap in terms of dietary macronutrient requirements across the culture period. While the nutritional needs of turbot have been investigated, the majority of that research has focused on the juvenile period (Cho et al., 2005; Sevgili et al., 2014b; Zeng et al., 2015; Ma et al., 2022; Pan et al., 2022). The studies during this early growth stage have suggested that turbot requires higher protein (>50%) (Caceres-Martinez et al., 1984; Li et al., 2011; Liu et al., 2015) but lower lipids (<13%) (Caceres-Martinez et al., 1984; Andersen & Alsted, 1993; Sevgili et al., 2014b; Zhang et al., 2015, 2022) and carbohydrates (ca. 15%) in their diets (Zeng et al., 2015; Miao et al., 2016; Zhang et al., 2019).

Large turbot weighing 660 g may not be able to withstand high dietary lipid levels (>15%), according to a previous study (Regost et al., 2001). However, a later study using fish of a similar size (580 g) suggested that fish may perform better on diets with lower protein (43.5%) and higher lipid (25.7%) (Leknes et al., 2012). Briefly, there is a clear discrepancy between the results of these studies in large turbot in terms of optimum balance between dietary protein and lipid. For this reason, further research into the interactive effects of macronutrients should be conducted to have a better understanding of their ideal nutritional needs.

The present study was planned to estimate the optimum dietary levels of proteins, lipids, and carbohydrates based on several zootechnical parameters and nutrient discharges in large- turbot based on a three-component mixture design.

Material and methods

Fish, rearing system and experimental design

The experiment was carried out at the Beymelek Unit of the Mediterranean Fisheries Research Production and Training Institute, Antalya (Türkiye). From the Central Fisheries Research Institute, Trabzon (Türkiye), juvenile turbots weighing approximately 25 g were moved and stocked into circular tanks with a water capacity of roughly 30 m^3. Each tank received brackish water (salinity range: 7.7 to 10.2 g/L) that had been UV (hot cathode lamb with a capacity of 40 mJ/cm^2, Wedeco Proxima, Aquada UV, Herford, Germany) treated.

After about a 2-month-acclimation, fish were size graded, transferred to 5 m^3 tanks and kept there until they weighed roughly 250 g. During the acclimatization of the fish to the conditions before the experiment, each of the 18 tanks, which measured 1.1 × 1.1 × 0.5 m and held 400 L of water, was randomly assigned to house a total of 18 fish.

The fish were then fed a commercial diet (Kılıç Deniz Ürünleri, Muğla, Türkiye) containing 54% protein and 12% lipid for 3 weeks. At the beginning of the experiment, the number of fish in each tank was reduced to 15 and their average weight was 301.6±0.1 g.

Fish were held in an indoor system that allowed natural light to enter and were subjected to a natural photoperiod of 10.5-13 hours light / 11-13.5 hours dark over the study period. Using a hand-held DO meter and pH meter (YSI Model 55 and 63, YSI Inc., Yellowsprings, OH, USA), water parameters including temperature, dissolved

oxygen, pH, and salinity were measured every day. The results were 19.28 ± 0.12 °C, 7.17 ± 0.06 mg/L, 7.51 ± 0.02 and 8.30±0.03 g/L, respectively. During the study, fish were collectively weighed at 3-week intervals after a one-day starvation period. The fish were fed their respective diets until apparent satiation by hand at 09:00 and 16:00 h for 10 weeks. At the beginning of the experiment, 10 fish were sampled to determine their initial body composition, while at the end of the experiment, four fish were sampled from each tank to determine their final body composition using an overdose of ethylene glycol monophenyl ether. Another four fishes were sampled to determine organo-somatic indices.

Experimental diets

To formulate the experimental diets, a mixture methodology using Scheffe cubic with a D-optimal design was employed because of the limited number of experimental tanks. The center point diet (K) was triplicated, and two additional diets (H and L) were duplicated, while other diets were tested in one tank. The main protein source was an equal blend of two fish meals, Danish LT and Domestic anchovy meal, based on the results of Sevgili et al. (2014a). Wheat flour and fish oil were used as the carbohydrate and lipid sources, respectively. To account for 93.85% of the diets, the minimum and maximum limits of fish meal (FM), wheat flour (W), and fish oil (FO) were set at 46.92-75.08%, 9.38-37.54% and 0.0-9.39%, respectively, as protein, carbohydrate, and lipid sources (Table 1). The mixture design model's ingredient limitations resulted in variations in protein levels ranging from 45.6% to 63.4%, carbohydrates ranging from 4.9% to 30.5%, and lipids ranging from 5.6% to 17.7% (Table 1). When setting the nutrient boundaries, the feeding habits of turbot (carnivorous) and the results of previous studies were considered. Each diet included an equal amount of wheat gluten meal (5%), vitamin mixture (0.25%), mineral mixture (0.1%), choline chloride (0.2%), and carboxymethyl cellulose (0.5%) (Table 2). The ingredients were ground using a hammer mill with an 800 µm screen (Kocamaz Machine, Model KT-20C, İzmir, Türkiye), weighed at predetermined levels, thoroughly mixed, pelleted using a pelleting machine (with a 9-mm die) without steam, packed in plastic bags, and stored at +4°C until use.

Calculations

$$\text{DFI g/kg MBW / day} = (\text{DMI} / \text{MBW}^{0.8}) / \text{day}$$

$$\text{MBW} = (\text{geometric mean of IW and FW})^{0.8}$$

$$\text{DGC} = [(\text{FW}^{1/3} - \text{IW}^{1/3}) / \text{day}] \times 100$$

Table 1. Formulation and nutrient compositions of experimental diets

Diets	Diet composition (%, as is basis)				No. of replicates	Nutrient composition (% of dry matter)							
	FM[2]	W[3]	FO[4]	Others[5]		DM[6]	Protein	Ash	Carb[7]	Lipids	GE[8]	C	C/N
A	75.08	18.77	0.00	6.15	1	92.7	63.4	15.7	14.6	6.3	20.0	44.7	5.1
B	66.08	27.77	0.00	6.15	1	92.9	57.1	14.1	23.0	5.7	19.8	44.7	5.7
C	75.08	12.21	6.56	6.15	1	93.8	62.5	15.4	8.2	13.9	21.7	47.3	5.5
D	75.08	15.99	2.78	6.15	1	93.5	61.5	15.4	13.5	9.6	20.7	45.8	5.4
E	46.92	37.54	9.39	6.15	1	93.4	45.6	10.5	29.0	15.0	21.7	48.5	7.8
F	67.08	17.38	9.39	6.15	1	93.5	56.2	14.0	12.8	17.0	22.2	48.4	6.3
G	63.81	27.72	2.32	6.15	1	92.0	57.2	13.9	20.3	8.6	20.4	45.8	5.8
H	54.91	31.86	7.08	6.15	2	93.5	50.4	11.8	24.8	13.1	21.4	47.7	6.9
I	75.08	9.38	9.39	6.15	1	93.7	62.2	15.1	4.9	17.7	22.6	48.7	5.7
J	56.31	37.54	0.00	6.15	1	92.0	51.6	12.3	30.5	5.6	19.7	45.0	6.4
K[1]	63.92	24.71	5.22	6.15	3	93.2	55.3	13.2	19.2	12.4	21.3	47.2	6.2
L	55.15	29.31	9.39	6.15	2	94.0	50.9	12.0	21.5	15.5	21.9	48.4	6.9
M	68.52	18.25	7.08	6.15	1	94.3	57.5	14.2	14.3	14.1	21.6	47.5	6.0
N	51.80	37.54	4.51	6.15	1	94.0	49.3	11.3	28.9	10.4	20.8	46.9	6.9

[1] Diet K is the center point with 3 replicates. [2] FM: fish meal, a 50%:50% mixture of Danish LT and domestic anchovy meal consists of 93.9% dry matter, 67.6% protein, 9.8% lipid, 14.8% ash and 19.6 MJ/kg gross energy (Sevgili et al., 2014a). [3] W: wheat flour, includes 89.6% dry matter, 15.9% protein, 4.4% lipid, 3.1% ash, 1.3% crude fiber, 65.0% carbohydrate and 16.4 MJ/kg gross energy (Sevgili et al., 2014a). [4] FO: fish oil. [5] Includes 5% wheat gluten (93.9% dry matter, 77.7% protein, 1.0% lipid, 0.9% ash, 0.5% crude fiber, 13.8% carbohydrate and 21.6 MJ/kg gross energy (Sevgili et al., 2014a)), 0.25% vitamin mixture, 0.1% mineral mixture, 0.2% choline chloride and 0.5% carboxymethyl cellulose. The vitamin and mineral mixtures are as reported previously (Akpınar et al., 2012; Sevgili et al., 2014a). [6] DM: dry matter. [7] Carb: carbohydrates. [8] GE: gross energy (MJ/kg)

Table 2. Growth, nutrient utilization and body indices of experimental fish

Diets	IW (g/fish)	FW (g/fish)	DGC (%/day)	DFI (g/kg $MBW^{0.8}$/day)	FCR	PER	CF	VSI (%)	HSI (%)
A	301.33	457.60	1.43	3.62	0.76	2.09	1.61	4.43	1.34
B	301.27	409.71	1.03	4.65	1.34	1.30	1.60	4.61	1.50
C	301.60	486.75	1.66	4.49	0.82	1.95	1.88	5.01	1.85
D	302.00	468.53	1.51	5.66	1.13	1.44	1.64	4.50	1.31
E	301.93	437.07	1.26	4.25	1.02	2.16	1.49	4.89	1.24
F	302.07	496.40	1.73	3.86	0.68	2.63	1.72	5.17	1.59
G	301.00	427.40	1.19	4.26	1.06	1.65	1.58	4.23	1.15
H	301.43±0.24	452.00±19.70	1.38±0.16	4.21±0.82	0.91±0.08	2.18±0.18	1.66±0.12	4.73±0.28	1.62±0.25
I	302.27	450.07	1.36	4.47	0.99	1.62	1.86	5.02	1.48
J	301.33	441.93	1.30	3.12	0.71	2.74	1.66	4.35	1.29
K[1]	302.16±0.10	444.44±16.01	1.31±0.13	4.60±0.58	1.05±0.16	1.74±0.26	1.63±0.03	4.85±0.26	1.41±0.03
L	301.10±0.05	454.03±12.78	1.40±0.10	3.68±0.52	0.80±0.17	2.51±0.54	1.66±0.08	4.79±0.79	1.50±0.28
M	302.07	490.53	1.68	4.40	0.80	2.18	1.60	4.92	1.43
N	300.93	508.50	1.83	4.20	0.70	2.91	1.67	5.03	1.48

[1] Diet K is the center point with 3 replicates and it is given as mean ± standard deviation to see the tank variation. IW: initial weight. FW: final weight. DGC: daily growth coefficient. DFI: daily feed intake. MBW: metabolic body. FCR: feed conversion ratio. PER: protein efficiency ratio. CF: condition factor. VSI: viscero-somatic index. HSI: hepato-somatic index.

FCR = DMI / weight gain

PER = weight gain / protein fed

CF = (average weight / standard length^3) × 100

VSI % = (visceral weight / body weight) × 100

HSI % = (liver weight / body weight) × 100

Daily nutrient intake (g kg $MBW^{0.8}$ day^{-1}) = [(N, lipid, energy intake / $MBW^{0.8}$) / days.

Daily nutrient gain (g kg $MBW^{0.8}day^{-1}$) = [(final body weight × final body nutrient) – (initial body weight × initial body nutrient)] / $MBW^{0.8}$ / days.

Nutrient retention (%) = 100 × (daily nutrient gain / daily nutrient intake).

N loss (g kg WG^{-1}) = (N intake – N deposited) / weight gain.

where: DFI = daily feed intake, MBW = metabolic body weight; DMI = dry matter intake; IW = initial weight; FW = final weight; DGC = daily growth coefficient; FCR = feed conversion ratio; PER = protein efficiency ratio; CF = condition factor; VSI = viscera somatic index; HSI = hepato-somatic index.

Chemical analysis

The fish samples were stored at -20 °C until analysis. Prior to analysis, they were thawed in a refrigerator and homogenized using a kitchen meat chopper (Tefal Le Hachoir 1500, France). Proximate analysis, with the exception of crude lipid, was performed on both the experimental diets and fish using the methods outlined by the AOAC (1990). Dry matter was determined by drying samples at 104 °C until a constant weight was achieved, whereas ash content was determined by incinerating samples in a muffle furnace at 600 °C for 2 hours. Crude protein (N×6.25) was determined by the Kjeldhal method after acid digestion. Finally, the lipid content was determined through ether extraction using an automatic extraction system (ANKOMXT15 Extractor, ANKOM Technology, Macedon, USA).

Statistical analysis

This study employed principal component analysis (PCA) to identify general relationships between variables and their directions. Data were analyzed using the statistical package JMP v.8.0 for Windows (SAS Institute Inc, Cary, NC, USA) in accordance with the mixture design methodology. The highest order models up to the cubic model using Scheffe polynomials were selected based on the p values of Lack of Fit and ANOVA and R^2. The main factors (FM, W and FO) were used regardless of the models selected, but effective terms were selected with a stepwise fit model based on the minimum Bayesian Information Criterion.

Optimum dietary macronutrient levels were estimated by examining the prediction profiler at the highest desirability that maximized selected variables, including final weight, DGC, PER, N, and energy retentions and minimized FCR, N, and C losses. Here, the optimization was performed by the highest desirability function of the target goals of the selected responses. Polynomial

contrasts up to the cubic level were also used to examine the relationship between DFI and dietary macronutrient concentrations.

Results and discussion

This study is the first to use a mixture design model to achieve balance among dietary protein, lipid, and carbohydrate concentrations in large turbot growing from 300 to 500 g. The model created 14 experimental diets with varying levels of FM, W, and FO, resulting in nutrient variations between 45.6-63.4% for proteins, 4.9-30.5% for carbohydrates, 5.6-17.7% for lipids with gross energy levels ranging from 19.7 to 22.6 MJ/kg, C levels ranging from 44.7% to 48.7 %, and C/N ratios ranging from 5.1 to 7.8 (Table 1). There were compensatory influences of changing dietary ingredients in terms of untargeted nutrients due to the use of intact macronutrient sources, FM and W, instead of purified ingredients such as casein and starch. Expectedly, for instance, dietary lipids of diets A, B, and J including FM and W at opposite amounts without FO reduced from 6.3% to only 5.6% because of lipids from increasing levels of W. The maximum dietary lipid and C/N ratio border in the present study was kept slightly lower than in our previous experiment (Sevgili et al., 2014b) considering its and other studies' results (Caceres-Martinez et al., 1984; Regost et al., 2001; Sevgili et al., 2014b; Zhang et al., 2022). The study showed that turbot fed experimental diets in limited tanks based on the mixture design model had good growth rates, feed utilization, and limited environmental impacts, which will be discussed further below.

Growth, nutrient utilization, and whole-body composition

Growth and feed utilization performance and organ-body indices are shown in Table 2, whereas whole body compositions and nutrient mass balance values are shown in Tables 3, 4 and 5, respectively. Fish grew from 300 g to a maximum weight of 509 g on experimental diets at the end of the study (Table 2). The center point (diet K) tested in the three tanks yielded an acceptable variation in the mean final weight and FCR, enabling reliable evaluation of the mixture design analysis (Fig. 1). Additionally, the Levene test confirmed the homogeneity of variance among the replicated treatments (diets H, K and L) in terms of final weight ($p=0.751$) and FCR values ($p=0.596$). The maximum growth performance was observed in fish on diet N (1.83 %/day of DGC) containing 49.3% protein, 28.9% carbohydrate, and 10.4% lipid, whereas the lowest growth was observed in fish on diet B (DGC of 1.03%/day), which contained 57.1% protein, 23.0% carbohydrate, and 5.7% lipid.

Even though the fish development rate in this study was in line with that of earlier research on large turbot (Regost et al., 2001; Leknes et al., 2012; Sevgili et al., 2012, 2021), a notable inter-treatment variation was observed among the nutrient concentrations considered. FCR values varied between 0.68 in diet F (56.2% protein, 12.8% carbohydrate, and 17.0% lipid) and 1.30 in diet B, consistent with those recorded in turbot in former studies (Regost et al., 2001; Leknes et al., 2012; Sevgili et al., 2012, 2021). The least consumed diet (3.12 g/kg $MBW^{0.8}$/day) by turbot was diet J, containing 51.6% protein, 12.3 % lipid, and 30.5% carbohydrate, whereas the highest consumption (5.66 g/kg $MBW^{0.8}$/day) was observed in diet D consisting of 61.5%

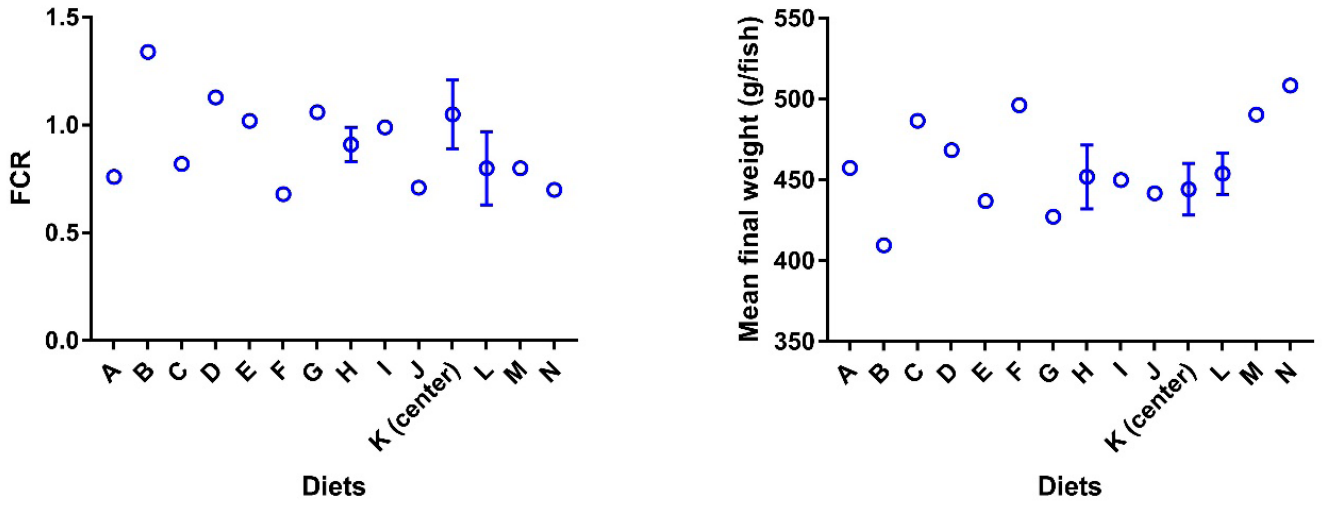

Figure 1. Mean final weight and feed conversion ratio (FCR) values of turbot fed experimental treatments. Mean values of diets H, K and L are shown as mean ± standard deviation.

Table 3. Whole body proximate compositions of fish fed varying experimental diets.

	Dry matter (%)	Ash (%)	Protein (%)	Lipid (%)	Gross energy (MJ/kg)	C (%)	C/N
Initial	22.94	4.70	15.08	1.90	4.32	9.41	4.55
A	23.48	4.61	16.03	1.65	4.45	9.72	4.42
B	22.05	4.46	15.35	2.51	3.92	10.02	4.76
C	23.74	3.86	15.85	2.79	4.86	10.50	4.83
D	23.52	4.13	15.76	2.36	4.67	10.12	4.68
E	24.22	4.60	15.28	3.08	4.84	10.43	4.97
F	24.60	4.49	15.30	3.86	5.15	11.05	5.26
G	23.90	4.85	16.77	2.74	4.40	10.95	4.76
H	24.27±0.69	4.28±0.13	16.56±1.99	2.72±0.57	5.00±0.25	10.82±0.60	4.78±0.31
I	23.74	4.08	15.20	3.72	5.07	10.88	5.22
J	22.94	4.48	15.68	1.54	4.32	9.44	4.39
K	23.73±0.95	4.12±0.07	15.29±0.60	2.63±0.32	4.66±0.24	10.09±0.51	4.81±0.10
L	24.32±0.16	4.14±0.15	16.23±0.74	3.08±0.63	5.06±0.07	10.93±0.10	4.91±0.27
M	24.26	4.34	15.45	3.31	4.97	10.70	5.05
N	23.72	4.24	15.70	2.02	4.52	9.83	4.56

protein, 15.4% lipid, and 13.5% carbohydrate. The DFI values fell within the range of those reported in previous studies (Sevgili et al., 2012, 2021). In the present study, no linear relationship was observed between DFI values and dietary macronutrient compositions, but significant linear, quadratic, and cubic trends ($p<0.05$) were observed when testing the impact of dietary protein levels using polynomial contrasts, indicating that an interaction between dietary protein and dietary carbohydrate or lipid concentrations was present. However, such a significant trend for DFI was not observed for dietary carbohydrate and lipid levels ($p>0.05$). Although dietary energy content is known to be the primary factor regulating feed intake in fish, it was not observed in the present experiment, likely because of the narrow range of the boundary. Instead, dietary protein levels appeared to play a more significant role in the voluntary feed intake in turbot. This finding is partly in line with that of Saravanan et al. (2012), who found an opposite relationship between feed intake levels and dietary digestible protein content in Nile tilapia, *Oreochromis niloticus*.

Dietary protein, carbohydrate, and lipid levels were located on different sides of the biplot by the first two PCAs (Fig. 2). Lipid was separated from other macronutrients by PCA1, whereas dietary protein and carbohydrate were on opposite sides of PCA2. The PCA also revealed that growth performance in turbot was strongly associated with the retentions of C and energy as well as N and energy gains, dietary lipid, and energy levels on the PCA1 axis (Fig. 2). These findings contradict many previous studies on juvenile turbot, which have shown that high dietary lipid levels can lead to reduced growth and nutrient retentions (Caceres-Martinez et al., 1984; Andersen & Alsted, 1993; Sevgili et al., 2014b; Zhang et al., 2015). Turbot are lean fish and have limited ability to tolerate and deposit excessive dietary lipid levels (Regost et al., 2001; Sevgili et al., 2014b; Zhang et al., 2022). However, as the fish grow, they appear to better utilize lipids by promoting the fatty acid β-oxidation (Zhang et al., 2022). The latter researchers showed that larger turbot (80 g) showed comparable expressions of lipolysis-related genes in the liver (pparα and cpt1) when they were fed increased dietary lipid (17.1%), which was not opposed to the findings in smaller fish groups (9 and 50 g). These findings are consistent with those reported for larger turbot (>500 g) by Leknes et al. (2012), who claimed that those fish showed optimum growth with a diet including 43.5% protein and 25.7% lipid. Accordingly, lipid retention values in the present study with larger fish were higher than those in our previous study with juvenile fish (54.4 g) when diets with similar compositions were compared (Sevgili et al., 2014b). Moreover, fish on diets E, F, I and L, including high lipid levels in the present study had remarkably lower energy and C retentions (average 35.8% and 34.6% respectively) (Table 5) than those recorded in the same study (Sevgili et al., 2014b) for diets with 16.0% and 18.6% lipid, which were 38.8% and 44.5%, respectively. These findings suggest that larger turbot are able to store more lipids in their bodies and better benefit from high dietary lipid contents for sparing amino acids as an energy source compared with their smaller counterparts.

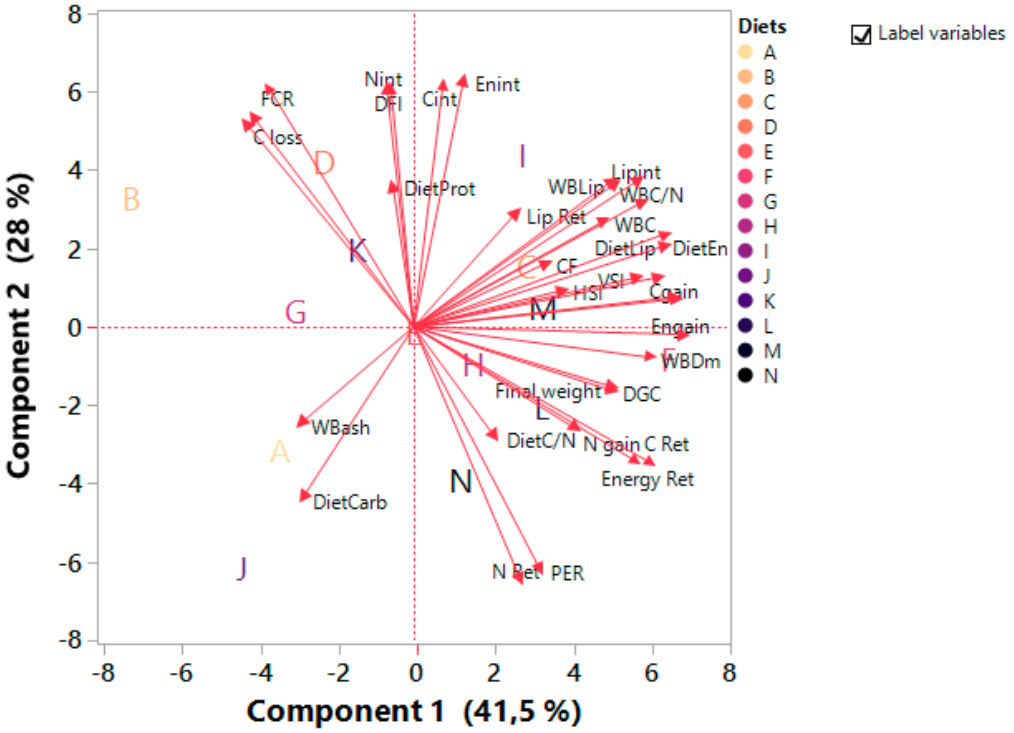

Figure 2. Projections of variables (arrows) and locations of experimental diets on the first two principal components. FCR: feed conversion ratio. C loss: carbon loss. N loss: nitrogen loss. Nint: nitrogen intake. DFI: daily feed intake. DietProt: dietary protein level. Cint: carbon intake. Enint: energy intake. Lip Ret: lipid retention. Lipint: lipid intake. WBC/N: whole body C/N. WBC: whole body carbon. CF: condition factor. DietLip: dietary lipid level. DietEn: dietary energy level. HIS: hepato-somatic index. VSI: viscerosomatic indekx. Cgain: carbon gain. Engain: energy gain. WBDm: whole body dry matter. DGC: daily growth coefficient. DietC/N: dietary C/N ratio. N gain: nitrogen gain. C Ret: carbon retention. Energy Ret: energy retention. PER: protein efficiency ratio. N Ret: nitrogen retention. DietCarb: dietary carbohydrate level. WBash: whole body ash.

Table 4. Nutrient intake and gains of fish fed fourteen different diets

Diet	Intake (g or kJ/kg MBW$^{0.8}$/day)				Gain (g or kJ/kg MBW$^{0.8}$/day)			
	Nitrogen	Lipid	Carbon	Energy	Nitrogen	Lipid	Carbon	Energy
A	0.37	0.23	1.62	72.46	0.14	0.06	0.51	23.17
B	0.43	0.27	2.08	91.97	0.09	0.15	0.42	10.00
C	0.45	0.62	2.12	97.43	0.16	0.24	0.70	32.61
D	0.56	0.54	2.59	117.08	0.14	0.17	0.59	27.53
E	0.31	0.64	2.06	92.15	0.11	0.25	0.55	25.97
F	0.35	0.65	1.87	85.78	0.15	0.41	0.81	38.18
G	0.39	0.37	1.95	87.04	0.14	0.19	0.60	18.83
H	0.34±0.07	0.55±0.11	2.01±0.39	90.12±17.62	0.15±0.06	0.21±0.07	0.65±0.14	30.32±6.07
I	0.44	0.79	2.17	100.86	0.12	0.35	0.65	30.94
J	0.26	0.18	1.41	61.44	0.12	0.03	0.43	19.45
K	0.41±0.05	0.57±0.07	2.17±0.28	97.89±12.44	0.11±0.02	0.19±0.05	0.52±0.11	24.47±5.26
L	0.30±0.04	0.57±0.08	1.78±0.25	80.57±11.48	0.14±0.01	0.26±0.10	0.67±0.05	31.57±2.75
M	0.41	0.62	2.09	95.27	0.15	0.32	0.74	34.70
N	0.33	0.44	1.97	87.20	0.17	0.14	0.66	30.17

Table 5. Nutrient retention and loss of fish fed fourteen different diets

Diet	Retention (%)				Loss (g/kg weight gain)	
	Nitrogen	Lipid	Carbon	Energy	Nitrogen	Carbon
A	38.41	25.44	31.42	31.98	45.84	225.05
B	21.67	56.47	20.18	10.87	93.18	464.39
C	34.73	38.65	32.95	33.47	51.40	249.73
D	25.41	30.64	22.93	23.52	79.82	383.98
E	35.27	39.03	26.79	28.18	46.18	347.17
F	42.73	62.69	43.13	44.51	33.52	179.20
G	35.01	53.25	30.77	21.63	61.82	328.98
H	43.15±9.06	39.35±20.17	32.39±0.72	33.63±0.16	40.02±3.00	283.34±20.99
I	26.06	44.16	29.99	30.68	70.14	324.37
J	47.41	19.70	30.48	31.66	30.09	217.12
K	28.42±6.47	34.18±12.74	24.48±6.42	25.35±6.74	65.04±15.50	366.36±85.55
L	48.08±4.15	47.66±24.32	38.48±8.35	39.83±9.09	32.67±9.50	231.27±79.57
M	36.65	51.98	35.28	36.43	44.40	234.21
N	50.38	31.65	33.34	34.60	26.15	208.68

Dietary protein level was closely associated with DFI, C, N, and energy intakes, which further supports the abovementioned association between feed intake and dietary protein. However, the dietary protein was moderately related to the losses of C and N (Fig. 2).

Dietary carbohydrate was moderately related to N retention and PER, suggesting a partial protein-sparing effect of carbohydrates in turbot and being consistent with the results of other marine finfish species such as European seabass (*Dicentrarchus labrax*) (Pérez et al., 1997), Senegalese sole (*Solea senegalensis*) (Conde-Sieira et al., 2016), gilthead seabream (*Sparus aurata*) (Basto-Silva et al., 2022) but not with those of European whitefish (*Coregonus lavaretus*) (Ruohonen et al., 2003; Vielma et al., 2003) and lumpfish (*Cyclopterus lumpus*) (Hamre et al., 2022). Whole body lipid, C, C/N, and energy levels were closely associated with dietary lipid levels, which is consistent with previous findings on juvenile turbot and shi drum, *Umbrina cirrosa* (Akpınar et al., 2012; Sevgili et al., 2014b).

Mixture design results

In the present study, only significant responses with a p-value of 0.05 were considered in the mixture design analysis, as indicated in Table 6. The models were found to have good R^2 values (>0.65) and an insignificant lack of fit (p>0.05), which is a prerequisite for a reliable approach. The model coefficients, standard error, and p-values of the terms are given in Table 7. It was observed that not all terms of the regression model for final weight and DGC values were significant (p>0.05) such as FO, FM*FO,

Table 6. Best model, target and summary of fit the selected responses

Response	Model	Target	p value of lack of fit	p value of ANOVA	R^2
Final weight (g/fish	Scheffe polynomial	Maximize	0.554	0.016	0.807
DGC (%/day)	Scheffe polynomial	Maximize	0.565	0.017	0.804
FCR	Cubic	Minimize	0.562	0.040	0.646
PER	Cubic	Maximize	0.734	0.005	0.766
N retention (%)	Cubic	Maximize	0.748	0.011	0.727
Energy retention (%)	Cubic	Maximize	0.565	0.017	0.804
N loss (g/kg weight gain)	Cubic	Minimize	0.562	0.040	0.646
C loss (g/kg weight gain)	Cubic	Minimize	0.734	0.005	0.766

DGC: daily growth coefficient. FCR: feed conversion ratio. PER: protein efficiency ratio.

W*FO, FM*FO*(FM-FO), and FM*W*FO (shown in Tables 7 and 8). Accordingly, insignificant coefficients can be removed from the model, but the main components should be retained. Thus, the equations for the final weight and DGC can be expressed as follows:

$$\text{Final weight} = 586_{FM} + 9738_{W} - 24855_{FO} - 18672_{FM*W} + 10404_{FM*W*(FM-W)}$$

$$\text{DGC} = 2.53_{FM} + 73.4_{W} - 196_{FO} - 145_{FM*W} + 79.1_{FM*W*(FM-W)}$$

Model terms of the responses, including FCR, PER, N, and energy retentions, N loss, and C loss, were statistically significant ($p<0.05$) and their model equations are given below.

$$\text{FCR} = -2.58_{FM} - 23.0_{W} - 150_{FO} + 50.9_{FM*W} + 223_{FM*FO} + 506_{W*FO} - 707_{FM*W*FO}$$

$$\text{PER} = 9.20_{FM} + 56.5_{W} + 360_{FO} - 111_{FM*W} - 519_{FM*FO} - 1155_{W*FO} + 1573_{FM*W*FO}$$

$$\text{N retention (\%)} = 172_{FM} + 1034_{W} + 6879_{FO} - 2057_{FM*W} - 10046_{FM*FO} - 22026_{W*FO} + 30482_{FM*W*FO}$$

$$\text{Energy retention (\%)} = 164_{FM} + 895_{W} + 6362_{FO} - 1871_{FM*W} - 9199_{FM*FO} - 20187_{W*FO} + 28061_{FM*W*FO}$$

$$\text{N loss (g/ kg weight gain)} = -235_{FM} - 2080_{W} - 13379_{FO} + 4450_{FM*W} + 19900_{FM*FO} + 44724_{W*FO} - 63045_{FM*W*FO}$$

Table 7. Mixture model parameter estimates for growth performance variables

Terms	Final weight (g/fish)			DGC (%/day)			FCR			PER		
	Coefficient	SE	p	Coefficient	SE	p	Coefficient	SE	p	Coefficient	SE	p
FM	586	208	**0.020**	2.53	1.67	0.164	-2.58	1.04	**0.031**	9.20	2.15	**0.001**
W	9738	2721	**0.006**	73.4	21.8	**0.008**	-23.0	6.0	**0.003**	56.5	12.4	**0.001**
FO	-24855	26557	0.374	-196	213	0.383	-150	45	**0.007**	360	93	**0.003**
FM*W	-18672	5252	**0.006**	-145	42.2	**0.007**	50.9	13.1	**0.003**	-111	27	**0.002**
FM*FO	50554	49788	0.336	394	400	0.350	223	63.6	**0.005**	-519	131	**0.002**
W*FO	-18667	34079	0.597	-157	274	0.581	506	126	**0.002**	-1155	261	**0.001**
FM*W*(FM-W)	10404	4356	**0.041**	79.1	35.0	**0.050**	N	N	N	N	N	N
FM*FO*(FM-FO)	-32351	24600	0.221	-256	198	0.227	N	N	N	N	N	N
FM*W*FO	56114	31629	0.110	461	254	0.103	-707	180	**0.002**	1573	372	**0.001**
W*FO*(W-FO)	N	N	N	N	N	N	N	N	N	N	N	N

Bold figures show significant coefficients ($p<0.05$). FM: fish meal. W: wheat meal. FO: fish oil. SE: standard error. p: p value. DGC: daily growth coefficient. FCR: feed conversion ratio. PER: protein efficiency ratio. N: not selected with a stepwise fit model.

Table 8. Model parameter estimates for nutrient retention and loss variables

Terms	N Retention (%)			Energy Retention (%)			N Loss (g/ kg weight gain)			C Loss (g/ kg weight gain)		
	Coefficient	SE	p	Coefficient	SE	p	Coefficient	SE	p	Coefficient	SE	p
FM	172	45	**0.003**	164	37	**0.001**	-235	94	**0.029**	-1158	495	**0.039**
W	1034	262	**0.002**	895	215	**0.002**	-2080	541	**0.003**	-9508	2861	**0.007**
FO	6879	1971	**0.005**	6362	1622	**0.002**	-13379	4076	**0.007**	-67095	21539	**0.010**
FM*W	-2057	570	**0.004**	-1871	469	**0.002**	4450	1178	**0.003**	20897	6224	**0.006**
FM*FO	-10046	2763	**0.004**	-9199	2274	**0.002**	19900	5713	**0.005**	98606	30187	**0.008**
W*FO	-22026	5495	**0.002**	-20187	4523	**0.001**	44724	11364	**0.002**	218104	60046	**0.004**
FM*W*(FM-W)	N	N	N	N	N	N	N	N	N	N	N	N
FM*FO*(FM-FO)	N	N	N	N	N	N	N	N	N	N	N	N
FM*W*FO	30482	7841	**0.003**	28061	6454	**0.001**	-63045	16215	**0.003**	-299439	85677	**0.005**
W*FO*(W-FO)	N	N	N	N	N	N	N	N	N	N	N	N

Bold figures show significant coefficients ($p<0.05$). FM: fish meal. W: wheat meal. FO: fish oil. SE: standard error. p: p value. N: not selected with stepwise fit model.

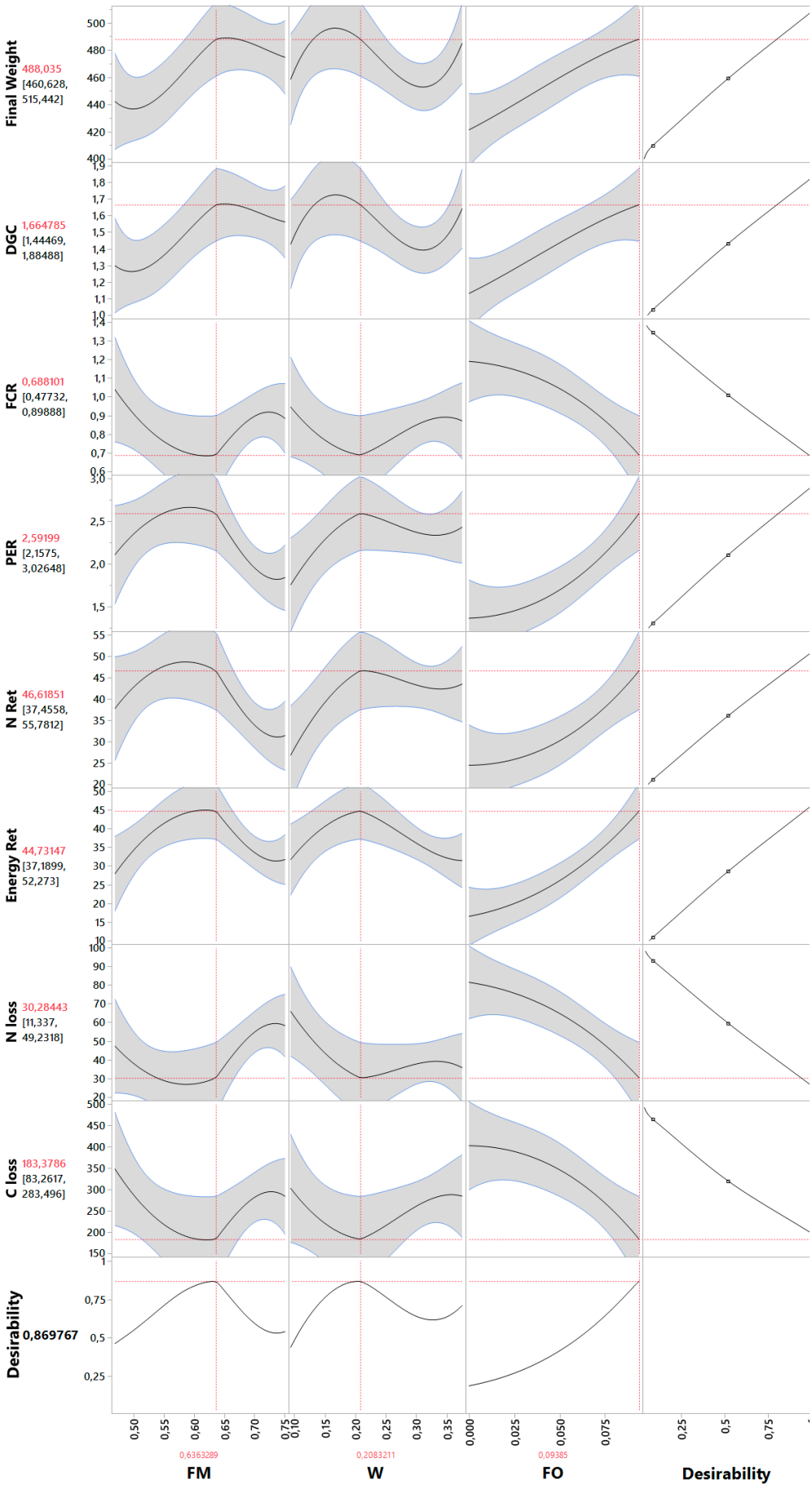

Figure 3. Prediction profiler of diet optimization with the goals of selected responses.

C loss (g/ kg weight gain) = $-1158_{FM} - 9508_{W} - 67095_{FO} + 20897_{FM*W} + 98606_{FM*FO} + 218104_{W*FO} - 299439_{FM*W*FO}$

In this study, the optimal formulation for large turbot diets was predicted by compromising the targets of each response (Fig. 3). The optimal formulation was based on the highest desirability (0.87) for all the selected response goals. The goals reached with the highest desirability were 488 g for final weight, 1.67% for DGC, 0.69 for FCR, 2.59 for PER, 46.7 % for N retention, 44.7% for energy retention, 30.3 g/kg weight gain for N loss, and 183 g/kg weight gain for C loss to the environment. The optimal dietary inclusion levels of FM, W, and FO were estimated to be 63.6, 20.8 and 9.4%, respectively (Fig. 3). Figure 4 shows the optimal dietary incorporation of main ingredients and the ternary plot of the selected responses. The resulting optimal dietary macronutrient compositions were calculated using the analysis values given in the footnote of Table 1 and were 49.68% protein, 15.62% lipid, and 14.46% carbohydrate as-is basis. The estimated dietary protein level was higher than what a previous study (Leknes et al., 2012) suggested as optimal (43.5%) for large turbot. Additionally, these researchers recommended increasing dietary lipid content to 25%, which is quite higher than our estimation. However, the maximum limit for dietary lipid inclusion in the current experiment was suggested by the mixture model was 17%, indicating that a somewhat higher upper limit must have been considered in the present study. The carbohydrate level is more or less consistent with the recommended level of 15% for juvenile turbot by Zeng et al. (2015), who addressed that fish can tolerate higher dietary carbohydrate levels and lower protein levels (about 46%) as water salinity increases. They also claimed that turbot should be reared at a salinity above 12 g/L, which is still higher than the salinity of the present study. Juvenile turbot showed better carbohydrate utilization and less influence from negative effects of its high dietary inclusions (>25%) when diets were supplemented with biotin (Liu et al., 2021; Pan et al., 2022) and taurine (Zhang et al., 2019). Therefore, it is difficult to argue that large turbot could utilize the dietary carbohydrate more effectively than the present study in higher salinity waters or with biotin and taurine supplementations, which clearly warrants further investigation. Further studies are clearly required to fully discover the nutrient requirements of turbot across the aquaculture stage, considering that the market size of turbot can be as high as 4 kg (Ruyet, 2002).

In conclusion, the results of the present study are significant from the perspective of filling the information gap about the optimum dietary macronutrient compositions of diets of large turbot. The results indicate that large turbot (300-500 g) require high dietary protein levels (54% dry matter basis) but can better utilize dietary lipid (>17% on dry matter basis) for energetic use than smaller juveniles. The specific diet formulation for this stage can also include 15.8% dietary carbohydrate on dry matter basis. The optimum nutrient concentrations will allow better diet formulations for this turbot size in terms of growth and nutrient utilization performance and environmental impacts.

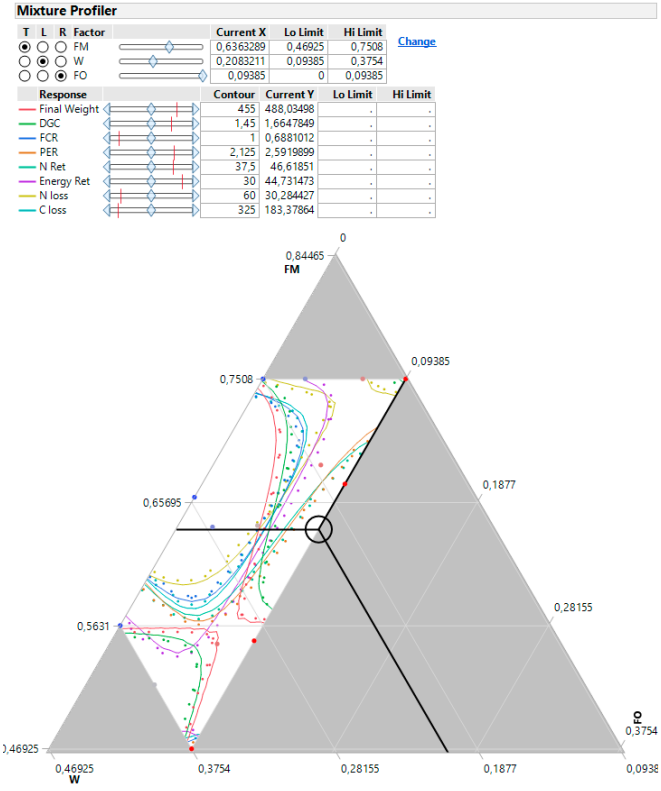

Figure 4. Ternary plot for the responses including final weight, DGC, FCR, PER, N retention, energy retention, N and C losses. FM: fish meal. T: top. L: left. R: right. W: wheat meal. FO: fish oil. DGC: daily growth coefficient. FCR: feed conversion ratio. PER: protein efficiency ratio.

Ethical approval: This experiment was carried out in accordance with the guidelines for the care and welfare of the principles of the European Directive (2010/63/EU) and the recommendations of the ARRIVE guidelines (Kilkenny et al., 2010).

Data availability: Data will be available on request.

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Authors' contributions: Hüseyin Sevgili: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Adem Kurtoglu**: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Masahiko Oikawa**: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Abdulkerim Aksoy**: Investigation, Writing – original draft, Writing – review

& editing. **Ramazan Uysal**: Formal analysis, Writing – original draft, Writing – review & editing. **Seçil T. Dugan**: Formal analysis, Writing – original draft, Writing – review & editing.

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